# Transgenic mouse analysis of *Sry* expression during the pre- and peri-implantation stage

David W. Silversides[1*], Diana L. Raiwet[1], Ouliana Souchkova[2], Robert S. Viger[3], Nicolas Pilon[2*]

[1] Department of Veterinary Biomedicine, Centre de recherche en reproduction animale (CRRA), Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, QC, Canada, J2S 7C6.
[2] Molecular Genetics of Development Laboratory, Department of Biological Sciences and BioMed Research Center, Faculty of Sciences, University of Quebec at Montreal (UQAM), 141 President-Kennedy Ave, Montreal, PQ, Canada, H2X 3Y7.
[3] Reproduction, Perinatal and Child Health, Centre de Recherche du Centre Hospitalier Universitaire de Québec (CRCHUQ), Centre de Recherche en Biologie de la Reproduction (CRBR) and Department of Obstetrics and Gynecology, Faculty of Medicine, Laval University, Quebec City, QC, Canada, G1V4G2

*co-corresponding authors:
David W. Silversides email: david.w.silversides@umontreal.ca
Nicolas Pilon email: pilon.nicolas@uqam.ca

BACKGROUND: The *SRY/Sry* gene is expressed in pre-Sertoli cells of the male genital ridge and functions as the mammalian testis determining factor (TDF). In addition, expression of *SRY/Sry* outside the genital ridge has been reported, including pre-implantation embryos, although the functional significance of this is not well understood.
RESULTS: Using Cre-mediated lineage studies and transgenic reporter mouse models, we now show that promoter sequences of human, pig and mouse *SRY* drive robust reporter gene expression in epiblast cells of peri-implantation embryos between e4.5 and e6.5. Analysis of endogenous *Sry* expression revealed that linear transcripts are produced via multiple polyadenylation sites in e4.5 embryos. Within the epiblast, *SRY* reporter expression mimics the expression seen using a *Gata4* reporter model, but is dissimilar to that seen using an *Oct4* reporter model. In addition, we report that overexpression of mouse *Sry* in ES cells leads to downregulation of the core pluripotency markers *Sox2* and *Nanog*.
CONCLUSION: We propose that *SRY/Sry* may function as a male specific maturation factor in the peri-implantation mammalian embryo, providing a genetic mechanism to help explain the observation that male embryos are developmentally more advanced compared to female embryos, and suggesting a role for SRY beyond that of TDF.

Key words: *SRY*, promoter, blastocyst, epiblast, male specific maturation factor, pluripotency, *Sox2*, *Oct4, Nanog*.

## INTRODUCTION

The SOX family of DNA binding proteins are a developmentally important family of vertebrate nuclear proteins involved in maintaining stem cell populations, making cell fate decisions, and contributing to cell and tissue differentiation (Harley and Lefebvre, 2010). In human and mouse, there are 20 *SOX* genes that can be organized by homology into eight families, designated *SOX A* to *SOX H* (Bowles et al., 2000; Koopman et al., 2004). All SOX proteins have a single 80 amino acid DNA binding motif known as a high-mobility-group (HMG) box domain that confers low affinity binding to DNA at a rather low specificity consensus sequence [(A/T)(A/T)CAA(A/T)G] (Harley and Lefebvre, 2010). Notably, binding of the HMG-box domain is to the minor groove of DNA, forcing the DNA molecule to bend in a characteristic fashion. The HMG box domain can also bind transcription factors such as POU and PAX members (Yuan et al., 1995; Kamachi et al., 2001; Remenyi et al., 2003; Wissmuller et al., 2006). For example, SOX2 can bind the POU factor OCT4 (POU5F1) in embryonic stem (ES) cells (Yuan et al., 1995) or with PAX6 in lens cells (Kamachi et al., 2001). Outside of the HMG box domain many SOX proteins have transcriptional activator domains, several have transcriptional repressor domains, while SOX D and SOX E family members have homo-dimerization domains (Kiefer, 2007). Cell specificity of SOX protein function is conferred at several levels, including tissue specific expression as defined by promoter elements, the presence (or absence) of competing (or collaborating) factors within the cell type in question, and post translational modifications of the SOX protein itself. Since all SOX proteins bind to the same DNA consensus sequences, if two or more SOX genes are expressed within the same cell type, there can be competition and/or compensation between SOX proteins for binding the same sites on target genes. For example, in oligodendrocytes, SOX D proteins (SOX5 and 6) compete with SOX E family members (SOX9 and 10) for SOX binding sites in the promoters of the *MPZ* and *MBP* genes (Stolt et al., 2006).

*SRY* (*Sex determining region Y*) was the first *SOX* gene identified (Sinclair et al., 1990), and conferred its name to the family (SOX: *SRY*-related HMG bOX). *SRY* is found on the mammalian Y chromosome and via homology is most closely related to SOX B1 members (SOX1/2/3) (Bowles et al., 2000). *SRY* is believed to have evolved from *SOX3,* itself located on the X chromosome (Waters et al., 2007), and SOX3 can substitute for SRY in transgenic experiments (Sutton et al., 2011). *SRY* functions in mammalian sex determination as the testis determination factor (TDF) (Koopman et al., 1991). It is expressed in male embryos within a sub-population of somatic cells of the indifferent genital ridge (Hanley et al., 2000; Bullejos and Koopman, 2001), thus defining the fate of these cells as pre-Sertoli cells and triggering the differentiation of the bi-potential genital ridge in the direction of a testis. However, *SRY* is not a typical SOX family member. Whereas most *SOX* members are represented broadly across vertebrates and invertebrates, *SRY* is a young gene found only in therian mammals (Veyrunes et al., 2008). Moreover, whereas most *SOX* orthologs have conserved sequences outside of the HMG box domain, both between species and across phyla (Bowles et al., 2000), only the HMG-

box sequences of *SRY* are conserved between mammalian species, with little to no structural conservation found outside the HMG box. Virtually all documented functional mutations of human *SRY* occur within the HMG box coding sequences (Cameron and Sinclair, 1997). In addition, it has been reported that goat *SRY*, when introduced into XX mouse embryos as a transgene, is able to induce testicular development (Pannetier et al., 2006); it is noteworthy that there is no sequence conservation between mouse and goat *SRY* outside of the HMG box. Taken together, these observations strongly suggest that SRY accomplishes its function as TDF via its HMG box only.

Although no other functions beyond TDF are documented for the *SRY* gene, extra-genital ridge expression of *SRY* has been reported in a variety of tissues and species. *SRY* expression is reported in the adult testes of several species (Hacker et al., 1995; Salas-Cortes et al., 1999), in both germ cells and Sertoli cells. *SRY* expression is also reported in the brain of human and mouse (Clepet et al., 1993; Harry et al., 1995; Lahr et al., 1995; Mayer et al., 1998; Mayer et al., 2000) and it was shown that *SRY* loss-of-function in brain tissues results in altered behavior in rats (Dewing et al., 2006). In addition, *SRY* expression is reported in the rat adrenal gland (Turner et al., 2007) and *SRY* gain-of-function in this tissue results in hypertension (Ely et al., 2007). In both brain and adrenal gland, *SRY* is believed to act upstream of the tyrosine hydroxylase gene and thus modulate the dopamine and norepinephrine biosynthesis pathway (Dewing et al., 2006; Ely et al., 2007). *SRY* expression is also reported in the healthy and neoplastic prostate gland (Tricoli et al., 1993; Lau and Zhang, 2000; Dasari et al., 2001). Finally, *SRY* expression is reported in pre-implantation blastocysts of several species, including human (Ao et al., 1994; Fiddler et al., 1995), mouse (Zwingman et al., 1993; Boyer and Erickson, 1994; Cao et al., 1995) and bovine (Gutierrez-Adan et al., 1997). The functional significance of these observations is currently not clear.

We have previously shown that human and pig *SRY* promoter sequences drive reporter gene expression within the mouse genital ridge at the time of sex determination (Daneau et al., 2002; Boyer et al., 2006). Furthermore, we reported that the human and pig *SRY* promoter sequences contain information that can also direct reporter gene expression to migrating neural crest cells (Boyer et al., 2006). We now report that human, pig as well as mouse *SRY* promoter sequences can in addition direct reporter gene expression to cells of the epiblast of the peri-implantation embryo. We confirm endogenous *Sry* expression in the e4.5 mouse embryo and identify novel poly-adenylation sites for *Sry* mRNA. We also show that *SRY* reporter gene expression in the epiplast is similar to what is seen for a *Gata4* reporter transgene whereas it appears complementary to the expression pattern of an *Oct4* reporter transgene. Semi-quantitative RT-PCR data further indicate that transient overexpression of *Sry* in ES cells results in reduced expression of the core pluripotency markers *Sox2* and *Nanog*. Consequently, we propose a model whereby expression of *SRY/Sry* in the epiblast may influence, in a sex specific fashion, the development of the pre- and peri-implantation mammalian embryo.

## RESULTS

***The SRY promoter can be active in epiblast cells of the pre- and peri-implantation embryo.***

In order to generate a Cre recombinase mouse line useful for targeted gene excision within pre-Sertoli cells of the bipotential genital ridge and given the specific activity of the pig *SRY* [4.6kb] promoter in these cells (Daneau et al., 2002), we generated three transgenic mouse lines using a Cre expression construct driven by the pig *SRY* [4.6kb] promoter. To test for Cre recombinase activity, we then mated these p*SRY*p[4.6kb]-Cre lines to the Cre reporter mouse line R26R-YFP (Srinivas et al., 2001). This reporter mouse line is particularly useful for cell lineage studies because, in the R26R-YFP background, cells that express Cre recombinase as well as their progeny will display fluorescence (Srinivas et al., 2001; Pilon et al., 2008).

At the time of birth, double transgenic animals (p*SRY*p[4.6kb]-Cre::R26R-YFP) showed evidence for ubiquitous Cre recombinase expression. For example, the testes showed extensive fluorescence but no distinct cell specificity (Fig. 1A, A'), and Cre activity could even be demonstrated by observing fluorescence in the skin (Fig. 1B, B'). One line was selected for more detailed analysis of the developing embryo. At e12.5, the genital ridges of both male and female double transgenic embryos displayed extensive fluorescence (Fig. 1C, C'). Fluorescence was additionally observed throughout the e9.5 embryo (Fig. 1D, D').

The extensive tissue distribution of Cre activity in newborn pups and in the e12.5 and e9.5 embryos suggested that the 4.6 kb pig *SRY* promoter is active in an early embryonic cell population. To identify this early embryonic cell population, p*SRY*p[4.6kb]-Cre::R26R-YFP double transgenic embryos were collected at e6.5 and e5.5. At these stages, fluorescence was seen in cells of the epiblast (Fig. 2). In double transgenic embryos at e4.5, faint fluorescence was also inconsistently seen within cells of the inner cell mass. Given that our cell lineage model needs the sequential activation of two proteins to detect *SRY* promoter activity, these observations are in accordance with the previous description of *SRY/Sry* expression in pre-implantation blastocysts (Zwingman et al., 1993; Ao et al., 1994; Boyer and Erickson, 1994; Cao et al., 1995; Fiddler et al., 1995; Gutierrez-Adan et al., 1997). It is also interesting to note that this pattern is similar to what we have previously observed with a $Gata4^{Cre}$ knockin allele (Pilon et al., 2008).

Our cell lineage data suggested that the enhancer sequences necessary for *SRY/Sry* expression in the pre-implantation blastocyst are contained within its proximal promoter. To more directly assess this possibility, we then looked at early embryos from our human and pig *SRY* reporter mouse lines (Daneau et al., 2002; Boyer et al., 2006). The human *SRY* promoter (h*SRY*p[5 Kb]-YFP) transgene was observed to support YFP expression within the epiblast of embryos from e4.5 to e6.5 (Fig. 3). At these stages, results were very similar to what was seen using the Cre recombinase model (Fig. 2) with the exception that fluorescence was now more consistently seen within the inner cell mass of e4.5 embryos. It was further noted that, in e4.5

embryos, the inner cell mass showed mosaic cell expression of fluorescence: some cells express fluorescence strongly while other cells express fluorescence moderately or not at all (Fig. 3B). This pattern of mosaic cell expression was still evident at e5.5 (Fig. 3E) and at e6.5 (Fig. 3H).

Three additional mouse lines, containing pig and/or mouse *SRY* fluorescence reporter transgenes, similarly supported reporter expression within epiblast cells of peri-implantation embryos (Fig. 4). The same 4.6 kb of pig *SRY* promoter used to drive Cre recombinase, when now used to drive GFP marker protein sequences directly (p*SRY*p[4.6kb]-GFP), resulted in fluorescence in cells of the epiblast in a similar fashion to what was seen using the human *SRY* promoter sequences (Fig.4A-C). A shorter pig *SRY* promoter (p*SRY*p[1.4kb]-YFP) gave a similar pattern of fluorescence (Fig.4D-F). Finally, we also observed that a transgenic mouse line containing a hybrid *SRY* promoter (-3 to -1 kb mouse, 1.4 kb pig) driving RFP (*SRY*p[hybrid]-RFP) (Boyer et al., 2006) also exhibited fluorescence within epiblast cells of e5.5 mouse embryos (Fig. 4G-I).

***In e4.5 embryos, linear Sry transcripts are produced via usage of multiple polyadenylation sites.***

In order to support our observations described above and further characterize the previously described *Sry* expression in the mouse blastocyst, we devised a RT-PCR approach for determining which of the two known poly-adenylation sites is involved at this stage (Hacker et al., 1995; Jeske et al., 1995). To this end, a single sided nested RT-PCR procedure was designed to overcome difficulties with working with mouse *Sry* transcripts (Fig. 5A-B). *Sry* is an intronless gene, so that genomic DNA and cDNA sequences are difficult to distinguish and contaminating genomic DNA can result in false positive results. To avoid problems with genomic DNA contamination, all RNA samples were treated with DNAse. The mouse *Sry* locus is flanked by inverted genomic repeat elements, and a feature of mouse *Sry* RNA is a circular, non-polyadenylated transcript that can be found in both adult and embryonic tissues including the blastocyst (Capel et al., 1993; Cao et al., 1995). To prevent PCR amplification from circular *Sry* transcripts, we performed the RT reaction using a poly-dT primer which should only prime polyadenylated RNA sequences, and then performed subsequent PCR reactions using non-specific reverse primers. As indicated in Fig. 5C, our analysis first revealed that a subset of *Sry* transcripts from e4.5 blastocysts, as well as those found in e11.5 genital ridges and adult testes, use the polyadenylation site previously reported by Jeske et al. (1995) at 3271 bp from the ATG start site. A novel polyadenylation site for these three tissues was also observed at 3015 bp from the ATG site. Moreover, for e4.5 embryos and adult testes, an additional novel polyadenylation site was observed at 2084 bp from the ATG start site. Of note, the site reported by Hacker et al. (1995), at 4468 from the ATG start site, was only observed in adult testes. Taken together, our data confirm that the *Sry* promoter is active in mouse blastocysts and further indicate that different linear transcripts (compatible with translation) are produced.

***At the pro-amniotic cavity stage of the peri-implantation embryo, male embryos tend to be developmentally more advanced compared to female embryos.***

Interestingly, from observing numerous e5.5 day embryos from the *SRY*p[hybrid]-RFP line, it became evident that within a given gestation, embryos could be slightly more (or less) advanced developmentally (data not shown). For example, the pro-amniotic cavity could be absent (indicating a younger embryo), present but not well developed, or present and well defined (indicating a slightly older embryo). Furthermore, we found that fluorescence peaked with the appearance of the pro-amniotic cavity and gradually became reduced with further embryo development. These observations raise the intriguing possibility that *SRY* reporters can be used as markers of maturation and/or early differentiation of the inner cell mass.

To verify this possibility, the *SRY*p[hybrid]-RFP reporter was used to study the well documented observations that male pre-implantation embryos are developmentally more advanced compared to female pre-implantation embryos (Tsunoda et al., 1985; Burgoyne, 1993; Cassar et al., 1994; Pergament et al., 1994; Cassar et al., 1995; Bernardi and Delouis, 1996). More precisely, the *SRY*p[hybrid]-RFP mouse line was used in a series of experiments to demonstrate that, at e5.5, embryos that were more developmentally advanced tended to be male (Fig. 6). In order to sex the embryos, we took advantage of a genetic marker consisting of an X chromosome integrated GFP transgene driven by 5 kb of the rat *Gata4* promoter (*Gata4*p[5kb]-GFP(2)) (Cory et al., 2007; Mazaud Guittot et al., 2007). Briefly, when this transgene is introduced from the paternal side, only XX embryos will receive it. Since this transgene generates discernable fluorescence within the pre-implantation embryo (Pilon et al., 2008), when this breeding strategy is followed, embryos at e5.5 that show GFP fluorescence are female. Based on morphological criteria (size, presence of pro-amniotic cavity, organization of the epiblast cell layer), embryos were ranked as being developmentally more or less advanced. From 7 gestations and 58 embryos total (of which 58.6% were male and 41.4% were female), 22 embryos were ranked as developmentally advanced. Of these, 18 were male and 4 were female. Interestingly, in early e5.5 gestations when developmentally advanced embryos are just beginning to form the pro-amniotic cavity and concomitantly show peak expression of the RFP fluorescence (due to the hybrid *SRY* promoter-RFP transgene), strong RFP fluorescence usually indicates male embryos. These observations support the idea that *SRY* reporters can be used as epiblast maturation/differentiation markers.

***Within the epiblast of the peri-implantation embryo, SRY and GATA4 reporters mark the same cell populations whereas SRY and OCT4 reporters mark different cell populations.***

To further confirm that *SRY* reporters are markers of maturation and early differentiation within the inner cell mass, mice homozygote for *SRY*p[hybrid]-RFP transgene were mated to mice homozygote for an autosomal *Gata4* reporter (*Gata4*p[5kb]-GFP(6a)), and embryos were collected at e5.5 of gestation. GATA4 and GATA6 are considered key regulators of early differentiation within the inner cell mass by triggering differentiation of the primitive endoderm

(Soudais et al., 1995; Fujikura et al., 2002; Chazaud et al., 2006) and we have recently found that the sequences necessary to drive expression of *Gata4* in the primitive endoderm are contained within its 5kb proximal promoter (Pilon et al., 2008). Interestingly, we have also found that such *Gata4* reporters additionally drive gene expression within epiblast cells not necessary committed to the primitive endoderm lineage although the functional significance of this observation is currently not known (Pilon et al., 2008). *SRYp*[hybrid]-RFP::*Gata4*p[5kb]-GFP(6a) double transgenic embryos were examined at e5.5 via confocal microscopy and it was observed that GFP and RFP emission patterns, when merged, mirrored each other in both distribution and relative intensity (Fig. 7A-D). This experiment was then repeated using two additional independent reporter mouse lines, to generate h*SRY*p[5 Kb]-YFP::*Gata4*p[5kb]-RFP(2) double transgenic embryos. When these embryos were examined at e5.5 via confocal microscopy, once again the GFP and RFP emission patterns mirrored each other (Fig. 7E-H). Thus, these observations are in accordance with the possibility that both promoters (*Gata4* and *Sry*) are upregulated in differentiating cells of the maturating epiblast.

The same breeding scheme was then used to verify if a converse relationship can be seen between the relative distribution of *SRY* promoter activity and a marker of pluripotency such as *Oct4* (Boyer et al., 2005). To this end, mice homozygote for the *SRY*p[hybrid]-RFP transgene were mated to mice homozygote for an *Oct4* promoter-GFP transgene (Anderson et al., 2000). Embryos were collected at e5.5 of gestation and examined via confocal microscopy (Fig. 8). It was now observed that GFP and RFP emission patterns in cells of the epiblast were mainly discordant in both distribution and relative intensity: a population of cells was predominantly green (*Oct4* promoter transgene dominance), a second population of cells was predominantly red (*SRY* promoter transgene dominance), while a third minority population of cells was yellow, indicating overlapping GFP and RFP emission patterns. Taken together, these observations suggest that, within the mammalian pre-implantation embryo, *SRY* and *Gata4* promoter activities (and by extension gene activities) share a developmental pathway which is divergent from a developmental pathway involving *Oct4* activity.

***Overexpression of Sry in ES cells leads to downregulation of pluripotency genes.***

The observations that the activity of the *SRY/Sry* promoter from multiple species correlates with early differentiation of epiblast cells suggest that the *SRY/Sry* gene product may be involved in this process by interfering with the activity of the closely related *SOX2/Sox2*. Indeed, SOX2 is a well-known stemness factor that forms, together with OCT4 and NANOG the core pluripotency network of ES cells (Boyer et al., 2005; Loh et al., 2006; Masui et al., 2007). Interestingly, within this network, these transcription factors are also known to regulate each other's expression via particular composite OCT-SOX enhancers in proximity to NANOG binding sites (Boyer et al., 2005; Masui et al., 2007; Chambers and Tomlinson, 2009). Thus, in order to gain insights into the putative role of *SRY/Sry* in the epiblast, we transiently overexpressed mouse *Sry* in undifferentiated R1 ES cells and assessed the expression levels of the pluripotency genes *Oct4*, *Sox2* and *Nanog* via semi-quantitative RT-PCR. To this end, we

also took advantage of a previously described bicistronic DomSry-IRES-GFP expression vector allowing FACS-mediated recovery of transfected cells (Lau and Li, 2009). As shown in Fig. 9, this analysis reveals that expression of *Oct4* is unaffected in ES cells grown under undifferentiating conditions but specifically overexpressing *Sry*. However, in the same conditions, expression of *Nanog* and *Sox2* is markedly reduced. These observations suggest that the Oct-Sox enhancer of the *Oct4* promoter may behave differently than the Oct-Sox enhancer of both the *Nanog* and *Sox2* promoter. Most importantly, they also strongly suggest that SRY/Sry proteins might be involved in epiblast differentiation at least by promoting exit of pluripotency.

## DISCUSSION

In mammals, there are examples of developmental sexual dimorphisms that are evident before gonadal sex determination and development (Erickson, 1997). Since these differences occur before the production of dimorphic sex steroids by the gonads, there must be a genetic rather than a hormonal explanation. An example of sexual dimorphism is the observation that male pre-implantation embryos are developmentally more advanced compared to female pre-implantation embryos. This was first reported in mice via classical re-implantation studies which showed that developmentally more advanced pre-implantation embryos were enriched for XY genotypes (Tsunoda et al., 1985; reviewed by Mittwoch, 1993). Follow up studies in the mouse (Burgoyne, 1993) confirmed that the male pre-implantation embryo could be developmentally more advanced and furthermore that this depended on the Y chromosome haplotype. *In vitro* work with bovine embryos demonstrated more rapid development of male pre-implantation embryos, including increased cell numbers and mitotic indices (Xu et al., 1992). *In vivo* studies of pig embryos showed that male embryos were developmentally more advanced compared to female embryos at days 5, 6 and 10 of pregnancy (Cassar et al., 1994; Cassar et al., 1995). In studies of human pre-implantation embryos fertilized *in vitro*, it was concluded that the presence of the Y chromosome causes accelerated growth rates immediately after fertilization (Pergament et al., 1994). Similarly, in *in vitro* fertilized and matured ovine embryos, fast developing embryos were found to be predominantly male (Bernardi and Delouis, 1996). At present, these observations are poorly understood at a mechanistic level. However, recent advances in the field of embryonic stem cells may now provide the basis for a mechanism to explain sex dimorphisms within the pre-implantation embryo.

Embryonic stem (ES) cells, whether of mouse or human origin, are pluripotent, non immortalized cells that can be grown in tissue culture and may be equivalent to epiblast cells of the mouse embryo, or primitive streak cells of the human embryo (Rossant, 2008). Understanding how these cells maintain their pluripotency and how they undergo differentiation has increased considerably in recent years. Indeed, this knowledge has been used to successfully reprogram differentiated somatic cells into induced pluripotent stem (iPS) cells displaying ES cell-like phenotypes (recently reviewed in Maury et al., 2012). The implication

of this work for understanding *in vivo* embryo development as well as for providing potential clinical applications has been noted (Jaenisch and Young, 2008; Rossant, 2008; Maury et al., 2012). A core regulatory circuitry of transcription factors within ES cells is proposed to maintain the pluripotent state, involving at a minimum the proteins OCT4, SOX2, and NANOG (Boyer et al., 2005; Loh et al., 2006; Masui et al., 2007). These factors interact with their own and each other's promoters, forming a self perpetuating auto-regulatory loop (Boyer et al., 2005; Chew et al., 2005; Chambers and Tomlinson, 2009). OCT4 and SOX2 proteins can physically dimerize with each other via their respective DNA binding domains and this interaction is thought to stabilize OCT4 binding on DNA (Remenyi et al., 2003; Williams et al., 2004; Chambers and Tomlinson, 2009). This SOX2/OCT4 dimer can activate several gene promoters, via binding of both proteins on a particular composite OCT-SOX element (Tomioka et al., 2002; Chambers and Tomlinson, 2009). The vast majority of the promoters regulated by this OCT-SOX element also contain a NANOG binding site in close proximity and these include the promoter of OCT4, SOX2 and NANOG themselves (Boyer et al., 2005). The autoregulatory loop formed by these three transcription factors is critically important for the stability of the self-renewal circuitry and, as such, is maintained within tight limits that can be subject to genetic or epigenetic perturbations. Slight increases or decreases in the expression of *OCT4, NANOG*, or *SOX2* can cause ES cells to exit the self-renewal circuit, thus triggering differentiation pathways (Fong et al., 2008; Kopp et al., 2008). Thus, *SOX2* is considered a stemness master gene (Harley and Lefebvre, 2010), helping to maintain stemness in ES cells and pluripotency in epiblast cells (Avilion et al., 2003). It is noteworthy that SOX2 can also maintain stem cell status during neurogenesis, and that decreased SOX2 levels results in increased nerve cell differentiation (Graham et al., 2003).

*SRY/Sry* expression has been reported in the pre-implantation embryo in several mammalian species (Zwingman et al., 1993; Ao et al., 1994; Boyer and Erickson, 1994; Cao et al., 1995; Fiddler et al., 1995; Gutierrez-Adan et al., 1997). We confirmed such observations for the mouse blastocyst and also found that different linear *Sry* transcripts compatible with translation are produced at this stage. Results from our *in vivo* cell lineage study using Cre recombinase under the control of 4.6 kb of pig *SRY* promoter sequences, although precluding the utility of this model for specific site directed excision in pre-Sertoli cells within the developing genital ridge, now provide further evidence that the *SRY* promoter can be active at an early stage in embryo development. Continued work with this model showed that, reminiscent of the pattern we have reported for $Gata4^{Cre}$-mediated lineage studies (Pilon et al., 2008), the *SRY* promoter is active within epiblast cells of the blastocyst stage embryo. Using transgenic fluorescence marker models, we confirmed that the human, pig and mouse *SRY* promoters contain sequence information that can support transcriptional activity within the inner cell mass and epiblast of the pre-implantation blastocyst, and that such transgene expression is possible in both male and female embryos. We have previously shown that the *SRY* promoter is synergistically responsive to GATA4 and WT1 levels (Miyamoto et al., 2008), and furthermore that *Gata4* is expressed within epiblast cells of the pre-implantation embryo (Pilon et al., 2008). *GATA4/Gata4* and

*WT1/Wt1* transcripts were also previously observed in undifferentiated human and mouse ES cells (Brandenberger et al., 2004; Wei et al., 2005; Pilon et al., 2008). Collectively, these observations suggest that transcription factors capable of driving *SRY* promoter activity are present within cells of the inner cell mass of both the male and female blastocyst stage embryos. Male specificity of *SRY* expression in the early embryo is obviously due to the fact that *SRY* is a Y chromosome located gene and, as such, is not found in XX embryos.

In mammals, it has been long observed that the male (XY) embryo tends to be developmentally more advanced in comparison to the female (XX) embryo (Mittwoch, 1993). Differences include embryo size, weight, number of somites, body length in post implantation embryos as well as cell number and stage of blastocyst maturity in pre-implantation embryos (Burgoyne, 1993). Burgoyne (1993) demonstrated that there is a positive Y chromosome effect on blastocyst cell numbers in mice, and furthermore that this effect can be strain and allele dependent. In the same year, Zwingman et al. (1993) reported that Y chromosome genes, including *Sry* and *Zfy*, are expressed within the male pre-implantation embryo as early as the 2 cell stage, and speculated that these factors may provide what has been called "growth factor Y". Further work by Burgoyne and coworkers (Thornhill and Burgoyne, 1993; Burgoyne et al., 1995) demonstrated, in post-implantation mouse embryos (e10.5), that a growth inhibiting X chromosome effect is present in XX embryos in addition to the growth accelerating effect of the Y chromosome seen in XY embryos. Using genetic models, these investigators were able to show that *Sry* is not responsible for the Y chromosome effect seen during this post-implantation time period. Although not supported by genetic models, it was further suggested that *Sry* would have little role to play in the Y chromosome effect observed during the pre-implantation of XY embryo. As suggested by Mittwoch (1993), we rather believe that the accelerated growth rate and increased maturity observed in male pre-implantation embryos is most likely due to a combination of genetic mechanisms. As recently presented by Arnold (2012), the sex chromosome complement can have differential effects on XX and XY embryos via several genetic mechanisms, including: male exclusive genes, X inactivation and dosage effects, X imprinting effects as well as X (or Y) heterochromatin sink effects (Arnold, 2012). Different combinations of these genetic effects could well be responsible for the differential growth and development seen in embryos of different ages, and it may not be prudent to equate post-implantation embryos with pre-implantation embryos.

Our present findings suggest that the potential role of *SRY* within the pre-implantation XY embryo should now be re-examined. In view of the importance of SOX2 levels for the maintenance of stem cell potential *in vitro* and for making early lineage fate decisions *in vivo* (Fong et al., 2008; Kopp et al., 2008), our data raise the possibility that the transcriptional activity of the closely related *SRY* within cells of the inner cell mass of pre-implantation male embryos may influence embryo development in a sexually dimorphic fashion. This possibility is supported by our semi-quantitative RT-PCR data and may provide a molecular explanation for the observations that pre-implantation male embryos in a number of mammalian species are

developmentally more advanced compared to their female counterparts (Tsunoda et al., 1985; Xu et al., 1992; Pergament et al., 1994; Cassar et al., 1995; Bernardi et al., 1996). All SOX proteins including SRY recognize the same core DNA binding motif. Thus, the SRY protein might compete with SOX2 via its HMG-box domain for the shared DNA binding sites of SOX proteins found within the promoters of SOX2 target genes which include the promoters of *OCT4*, *NANOG* and *SOX2* itself (Boyer et al., 2005; Loh et al., 2006). In agreement with this possibility, previous work with murine *Sox2*-null ES cells has shown that other Sox proteins expressed in ES cells (Sox4, Sox11 and Sox15) can also fulfill an activating role in the regulation of otherwise Sox2-regulated enhancers (Masui et al., 2007). Mechanistically, binding of SRY on such SOX elements might thus interfere with the positive SOX2 (and other SOX) activity either by passive competition or active repression (Lau and Li, 2009; Peng et al., 2009). Additionally, given the high homology of the HMG box domain between SRY and SOX2, SRY is also predicted to interact with OCT4 on composite OCT-SOX elements (Remenyi et al., 2003). This possibility is supported by ChIP-chip experiments performed in Ntera2 testicular embryonal carcinoma cells indicating that OCT4 and SRY colocalize on hundreds of target promoters (Jin et al., 2007). Interestingly, precedents for both of these mechanisms are also provided by the competitive inhibition of SOX E family proteins by SOX D family proteins in oligodendrocytes (Stolt et al., 2006).

In conclusion, the possibility that SRY has a biological function beyond TDF and sex determination, i.e. that of a genetic male specific maturation factor within cells of the inner cell mass of the pre- and peri-implantation blastocyst, provides new insights and raises new questions about this intriguing mammalian specific, male only molecule.

## EXPERIMENTAL PROCEDURES

### *Mouse lines and trials*

The production of mouse lines transgenic for the human *SRY* promoter [5 kb]-YFP, Hybrid *SRY* promoter-RFP, rat *Gata4* promoter [5 kb]-GFP with transgene integration on the X chromosome (used for sexing embryos), and rat *Gata4* promoter [5 kb]-RFP has been previously described (Boyer et al., 2006; Mazaud Guittot et al., 2007; Pilon et al., 2008). All mice used were of FVB/n genetic background. The Cre reporter R26R-YFP mouse line was originally provided by Frank Costantini (Srinivas et al., 2001); we subsequently placed the marker allele onto a FVB/n genetic background via backcrossing. Oct4-GFP marker mice (Anderson et al., 2000) were a gift from Chris Wylie; the Oct4-GFP marker transgene was introduced onto the FVB/n genetic background via backcrossing. The human *SRY* promoter [5 Kb]-YFP, *SRY* promoter [hybrid]-RFP, rat *Gata4* promoter [5 Kb]-RFP, and Oct4-GFP lines were all maintained as homozygous lines; thus appropriate matings for double marking trials resulted in all embryos being doubly heterozygote for the appropriate transgenes. For staged embryo collections, females were housed with fertile males, and noon on the day when a

seminal plug was observed was designated as e0.5. Embryos at e4.5 were collected by uterine flushing using PBS; embryos at later stages were recovered via dissection.

***Pig SRY promoter [4.6 kb]-Cre mouse***

Using standard molecular biology construction techniques, a pig *SRY* promoter [4.6 kb]-GFP genetic construction (Daneau et al., 2002) was modified such that the GFP coding sequence was replaced by Cre recombinase coding sequences (Boyer et al., 2002). The resulting transgene, designated p*SRY*p[4.6kb]-Cre, was microinjected into the male pronucleus of single celled FVB/n embryos, and transgenic mice were generated using standard methodologies (Daneau et al., 2002; Boyer et al., 2006). A tyrosinase minigene was co-injected to provide visual identification of transgenic mice. Pigmented founder animals were bred to FVB/n non-transgenic mice to insure single integration sites (Methot et al., 1995). Co-integration of Cre sequences was verified by PCR analysis on genomic DNA (data not shown). Three lines were retained and mated to the Cre reporter R26R-YFP mouse line; the activity of the Cre recombinase was verified by observing newborn tissues of double transgenic animals with fluorescence optics. All lines showed similar results; one line was retained for further studies.

***Microscope studies and imaging***

Newborn mice were photographed with a Leica MZFLIII stereomicroscope equipped with fluorescence excitation and filters for GFP capture. Image acquisition was via a Nikon DXM1200 digital camera, using Nikon Act-1 software. For visible light, fluorescence and confocal imaging, a Nikon Eclipse E800 microscope was used that was equipped for fluorescence excitation and with filters for GFP and RFP capture, and connected to a Nikon C1 confocal imaging system. The confocal system had an argon laser for excitation and filters for GFP and YFP emission capture, as well as a green helium-neon laser for excitation and filters for RFP emission capture.

***Mapping of Sry polyadenylation sites in e4.5 mouse embryos.***

For pre-implantation embryos, 232 day e4.5 embryos were collected by uterine flushing and processed without attempting to sex the embryos. For collecting e11.5 male genital ridges, matings were performed using a transgenic mouse line incorporating a *Gata4* promoter-GFP and tyrosinase transgenes incorporated on the X chromosome; sexing of embryos was performed by the presence in females (and absence in males) of fluorescence and pigmentation (Cory et al. 2007). Genital ridges from 34 male embryos at age e11.5 were dissected and pooled. For adult testes, tissues were homogenized using a Polytron PT1300D apparatus with a 5mm probe. For all tissues, total RNA was then extracted using a RNeasy mini kit (Qiagen). The samples were digested with DNAse enzyme, using RNase-free DNase Set (Qiagen), according to the supplier's instructions. Total RNA was then further processed to derive mRNA using a Oligotex Direct mini kit (Qiagen), again according to the manufacturer's instructions. Reverse transcription was performed using Omniscript RT enzyme (Qiagen), incubating samples at 37˚C for 1 hr, in the presence of a poly-dT primer (Reverse Primer 1, see Table1).

An anchored, nested PCR procedure was devised, as presented in Fig. 5. Specific forward nested primer triplets, and non-specific reverse primers, are presented in Table 1. All PCRs consisted of cycles of denaturation at 95˚C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72˚C for 90 sec. PCR reactions were performed in a total reaction volume of 100µl. A first PCR reaction of 20 cycles was performed using a specific forward primer and reverse primer 2. Five (5) µl of this completed reaction was used to seed the second reaction of 30 cycles using the first nested specific forward primer and reverse primer 3; 5µl of this completed reaction was used to seed the third reaction of 30 cycles using the second nested specific forward primer and reverse primer 3. PCR products were visualized on a 1% agarose gel with ethidium bromide. PCR products were then subcloned into pDrive plasmid vector (Qiagen), and plasmid miniprep DNA prepared and analyzed for insert size. Selected plasmids were sequenced and insert sequences used to search mouse genomic sequences using the Blastn function of Ensembl.org; positive hits for *Sry* sequences were recorded.

***Semi-quantitative RT-PCR assays in ES cells***

R1 ES cells were grown in standard ES cell medium containing LIF onto gelatin-coated dishes as previously described (Pilon et al., 2008). Approximately $10^5$ cells were transfected with 5µg of either DomSry-IRES-GFP or empty IRES-GFP control vector. Transfections were performed with Genejuice reagents (Novagen) in accordance with manufacturer's instruction and transfected cells were cultivated for 48h in complete ES cell medium. At the end of the cultivation period, cells were harvested and GFP-positive cells recovered by FACS. For each condition, mRNA was isolated from $3x10^4$ cells with the Oligotex Direct mRNA kit (Qiagen) and reverse transcription was performed with an oligodT primer and Superscript II reagents (Invitrogen). For PCR amplifications, with the sole exception of *Sox2*, primers were designed to encompass an intron, allowing the detection of contaminating genomic DNA by the presence of a larger band. Specific pairs of oligonucleotides and expected size of the amplicons are listed in Table 2. PCR amplifications were performed with Platinum Taq (Invitrogen) and consisted of 20 to 35 cycles of 35 sec at 96°C, 30 sec at 61°C, and 45 sec at 68°C. PCR products were size fractionated on a 1.5% agarose gel. PCR analyses were performed three times from three independent reverse transcriptions.

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## FIGURE LEGENDS

**Fig. 1. Pig *SRY* promoter sequences driving Cre recombinase results in indiscriminate tissue marking in cell lineage studies.** (A-D): visible light illumination; (A'-D'): epifluorescence illumination with GFP filters. **A:** Newborn testes at 40X magnification. Wild type (WT) on left, p*SRY*p[4.6kb]-Cre::R26R-YFP double transgenic on right. **B:** New born pups, view of head. Wild type (WT) pup on top, p*SRY*p[4.6kb]-Cre::R26R-YFP double transgenic pup on bottom. Image was taken at 8X magnification. **C:** Testes (T), ovaries (O) from p*SRY*p[4.6kb]-Cre::R26R-YFP double transgenic embryos at e12.5 (100X magnification). **D:** e9.5 embryo from p*SRY*p[4.6kb]-Cre::R26R-YFP cross. 40X magnification.

**Fig. 2. Pig *SRY* promoter sequences drive Cre recombinase expression in an early embryonic cell population.** Embryos are double transgenic (p*SRY*p[4.6kb]-Cre::R26R-YFP). (A-C): e6.5 gastrulation stage embryo, 200X magnification. (D-F): e5.5 epiblast stage embryo, 400X magnification. **A,D:** Visible light. **B,E:** GFP emission. **C:** Merge of A and B images. **F:** Merge of D and E images.

**Fig. 3. Human *SRY* promoter sequences drive reporter protein expression within cells of the pre- and peri-implantation embryo.** Transgene is h*SRY*p[5kb]-YFP. (A,D,G): visible light. (B,E,H): YFP emission. (C,F,I): visible light, YFP emission merge. **(A-C):** e4.5 pre-implantation embryo, 400X magnification. The outline of the inner cell mass is indicated by a red dotted line in A. In B and C, it is evident that some inner cell mass cells display strong fluorescence while others display no fluorescence. **(D-F):** e5.5 epiblast stage embryo, 400X. The epiblast is now organized (indicated by a red dotted line in D), and the pro-amniotic cavity has formed. Fluorescence displayed by epiblast cells is more uniform than seen in cells of the inner cell mass, but still shows variation in intensity between cells. **(G-I):** e6.5 gastrulation stage embryo, 200X. Fluorescence is quite variable between cells.

**Fig. 4. At e5.5, pig and mouse *SRY* promoter sequences drive reporter transgene expression in cells of the inner cell mass that resolve themselves into the epiblast.** Confocal optics at 400X magnification were used. (A,D,G): visible light. (B,E,H): fluorescence emission. (C,F,I): merged images of visible light, fluorescence emission. **(A-C):** p*SRY*p[4.6kb]-GFP transgene. The embryo depicted is at the early pro-amniotic cavity stage. The promoter sequence used is the same length that was used in the Cre-recombinase study and, at this stage, the two models give similar results. **(D-F):** p*SRY*p[1.4kb]-YFP transgene. Embryos 1 and 2 are at e5.5, and are both slightly more advanced in development than the embryo depicted in (A-C). Embryo 3 is at e4.5 and fluorescence is only just becoming evident. **(G-I):** *SRY*p[hybrid]-RFP transgene consisting of 1.4kb of pig and 2 kb of mouse promoter sequences. Fluorescence marking is similar to that seen in embryos with the h*SRY*p[5kb]-YFP, p*SRY*p[4.6kb]-GFP, and p*SRY*p[1.4kb]-YFP reporter transgenes at all stages, and to p*SRY*p[4.6kb]-Cre::R26R-YFP double transgenic embryos at e5.5.

**Fig.5. Mapping of *Sry* polyadenylation sites in e4.5 embryos**
**A:** Schematic presentation of the single sided, nested RT-PCR strategy for identifying endogenous *Sry* mRNA from e4.5 embryos, e11.5 male genital ridges and adult testes. F1, F2 and F3 represent the nested triplet of forward primers; R1(poly-dT) was used for reverse transcription, while R2 and R3 were used for anchored nested PCR amplifications
**B:** Polyadenylation site mapping of endogenous mouse *Sry* transcripts from non transgenic mice. Map of FVB/n mouse *Sry* gene including open reading frame and 5 Kb of 3' sequences (numbered from ATG start = 1). Nested triplet forward primers for 3'sequences of mouse *Sry* gene are marked (see Table 1), where numbering of primers refers to the 5' end of the first nested primer. Numbering below the horizontal line refer to sites where polyadenylation was observed by the RT-PCR and sequencing strategy. **C:** Sites at which polyadenylation was observed for the *Sry* transcript, correlated with tissues. The genomic sequences found just 5' to the polyadenylation sites are listed.

**Fig. 6. At e5.5, developmentally advanced embryos are biased towards males.** In this study, male mice transgenic for *Gata4* promoter-GFP(2) transgenes (with X-chromosome integration) were mated to homozygote *SRY*p[hybrid]-RFP females. Resulting embryos were observed at e5.5, with confocal optics at 200X magnification. Embryos from one gestation are shown. RFP emission marks the epiblast cells of XX and XY embryos; GFP emission marks the epiblast of XX embryos, and is used to sex embryos. Based on anatomic criteria (size of embryo, maturity of pro-amniotic cavity, organization of epiblast) as well as sexing, more advanced embryos are biased towards being male. **A:** Visible light. By physical size and maturity of the epiblast cell layer, embryos labeled 1 and 2 are the most mature. For embryos 3, 4 and 5, the pro-amniotic cavity is just becoming defined. **B:** RFP emission. Note that embryos 3, 4 and 5 show peak RFP emission, and are overexposed to show expression of embryo 1. **C:** GFP emissions, allowing sexing of embryos, since inner cell mass/epiblast cells of female embryos fluoresce. Embryos 3, 4 and 5 are female. **D:** Merged GFP, RFP emissions and visible light. The more developmentally advanced embryos (1, 2) are male.

**Fig. 7. At e5.5, *Gata4* and *SRY* reporter transgenes mark the same cell populations within the epiblast.** (A-D) *Gata4* promoter-GFP(6a)::*SRY*p[hybrid]-RFP double transgenic embryo, shown at a stage before the pro-amnionic cavity has formed. (E-H) h*SRY*p[5kb]-YFP::*Gata4* promoter-RFP(2) double transgenic embryo, shown at the early pro-amniotic cavity stage. For a given fluorescence marker, some cells of the epiblast are marked more strongly (or more weakly) by fluorescence. Between markers, the same cells are marked more strongly (or more weakly). Thus within the epiblast, the transgene marker pairs mark epiblast cells in a similar fashion. Images are taken with confocal optics at 400X magnification. **A,E:** Embryo with visible light. **B,F:** GFP emission. Note that all cells of the epiblast are green, but that the intensity of green between cells can be variable. **C,G:** RFP emission. All cells of the epiblast are red, although the intensity of red between cells can be variable. Note also that for the RFP

channel, particularly in (C), trophectoderm cells give emission; this was commonly seen irrespective of transgene. **D,H:** Merged GFP and RFP emission profiles. All cells of the epiblast are yellow; the intensity (but not the hue) of yellow can be variable.

**Fig. 8. At e5.5, *Oct4* and *SRY* reporter transgenes mark different cell populations within the epiblast.** Shown are *Oct4* promoter-GFP::*SRY*p[hybrid]-RFP transgenes in double transgenic embryos at e5.5. Green fluorescence is seen in cells of the epiblast that express the *Oct4* promoter-GFP reporter transgene; red fluorescence is seen in cells that express the *SRY*p[hybrid]-RFP reporter transgene. All images are at 400X magnification. (A-D): early e5.5, before the formation of the pro-amniotic cavity and full organization of the epiblast. (E-H): late e5.5, after formation of the pro-amniotic cavity and organization of the epiblast. **(A, E):** Visible light. **(B,F):** GFP emission. **(C,G):** RFP emission. **(D, H):** Merged fluorescence images show that there is a population of inner cell mass/epiblast cells that express predominantly the *Oct4* promoter transgene (green), cells that express both transgenes (yellow), and cells that express predominantly the *SRY*p[hybrid]-RFP transgene (red), indicating two populations of cells with some expression overlap.

**Fig. 9. Transient overexpression of *Sry* in ES cells results in reduced expression of pluripotency genes.** A semi-quantitative RT-PCR approach was used to evaluate the effect of *Sry* overexpression in ES cells on the expression levels of the core pluripotency markers *Oct4*, *Nanog* and *Sox2*. Amplification of *Gapdh* was used for normalization. Prior to RT-PCR analyses, murine R1 ES cells were transfected with DomSry-IRES-GFP (Sry) or empty IRES-GFP (Ctl) vector and GFP-positive cells recovered by FACS. In the presence of *Sry* overexpression, expression of *Nanog* and *Sox2* is clearly reduced whereas expression of *Oct4* appears unaffected. It is noteworthy that similar results were obtained from three independent RT-PCR assays.

**Data not shown: In e5.5 embryos, the *SRY*p[hybrid]-RFP transgene marks cells of the epiblast, with variable intensity displayed between embryos.** (A-C): Five embryos from a single gestation are shown at 200x. **A:** visible light. **B:** RFP emission. **C:** merge of images in A, B. Variable fluorescence signal intensity (stronger, weaker) is observed between embryos. Through observations of e5.5 stage embryos from numerous gestations, it was observed that intensity of fluorescence was maximal in embryos at the early pro-amniotic cavity stage, and declined subsequently.

**TABLE 1:** Primers used for mapping of *Sry* polyadenylation sites:

**Forward nested triplet primers I,J,C:**

**(F1):mSRY3'.i (**1920 bp from ATG) 5'-GGTTGAGCATTCTAATGACTGGG

**(F2):mSRY3'.j (**1961 bp from ATG) 5'-GAGCAGGCAAAAACACAGTGCC

**(F3):mSRY3'.C** (2157 bp from ATG) 5'-GGGTTTCTCTCTAGCACACAAAAC

**Forward nested triplet primers O,P,M:**

**(F1):mSRY3'.0 (**2562 bp from ATG) 5'-GTGGGATGAATGAATGGCCATCTGC

**(F2):mSRY3'.P (**2619 bp from ATG) 5'-GGAATGGTTCAAAGTTACCCACTC

**(F3):mSRY3'.M** (2757 bp from ATG) 5'-TACCCAGACCTTCAGTTAGCCTG

**Forward nested triplet primers Q,R,S:**

**(F1):mSRY3'.Q** (3051 bp from ATG) 5'-GTATTGTGAACAGTAACTTACATGTATTG

**(F2):mSRY3'.R (**3101 bp from ATG) 5'-GACTGTTCTCTAGAATTCAATGGAAC

**(F3):mSRY3'.S** (3128 bp from ATG) 5'-GTTAGCTGGCACTACTGGACTTC

**Forward nested triplet primers N,G,H:**

**(F1):mSRY3'.N (**4317 bp from ATG) 5'-ATGCTAACTCCCTACTGACCAGGG

**(F2):mSRY3'.G** (4454 bp from ATG) 5'-CCATTAGCAAAAAGTATTCCCTGGG

**(F3):mSRY3'.H (**4478 bp from ATG) 5'-ATACTGTTCTTCTGGAAAGCTTAC

**(R1):Reverse Primer 1 (poly-dT)** 5'-GGATCCAAGCTTGAATTCTAATACGACTCACTATAGGG$(T)_{17}$

**(R2):Reverse Primer 2** 5'-GGATCCAAGCTTGAATTCTAATACG

**(R3):Reverse Primer 3** 5'-GAATTCTAATACGACTCACTATAGGG

**TABLE 2. Oligonucleotide Pairs Used for RT-PCR Analyses**

| Gene | Forward primer | Reverse primer | Size (bp) |
|---|---|---|---|
| *Oct4* | 5'-GATGGCATACTGTGGACCTCAGGTTG | 5'-CTGATTGGCGATGTGAGTGATCTGC | 560 |
| *Sox2* | 5'-TGTCGCACATGTGAGGGCTGGAC | 5'-CGCCCTCAGGTTTTCTCTGTACAA | 278 |
| *Nanog* | 5'-TCTCCTCCATTCTGAACCTGAGCT | 5'-AAAGTCCTCCCCGAAGTTATGGAG | 380 |
| *Gapdh* | 5'-TCCTGCACCACCAACTGCTTAGC | 5'-AGGTCCACCACCCTGTTGCTGTA | 530 |

Figure 1

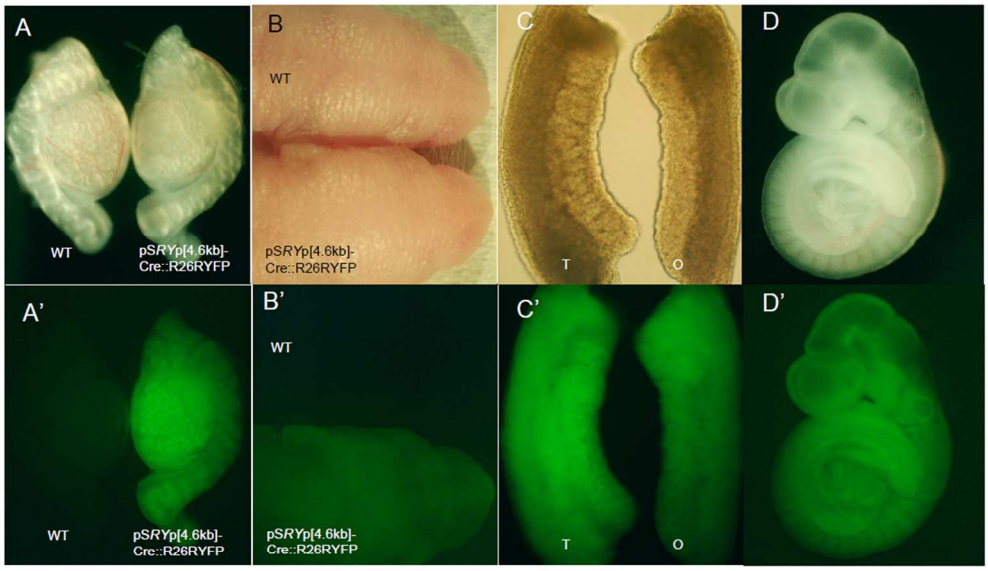


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Figure 2

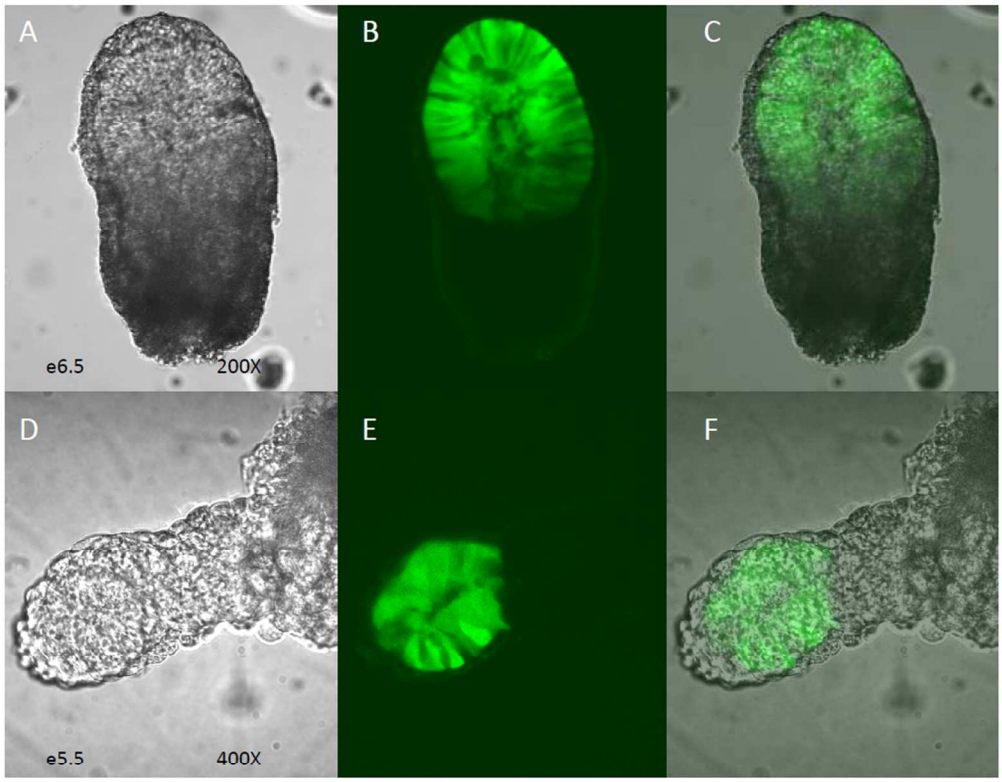


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Figure 3

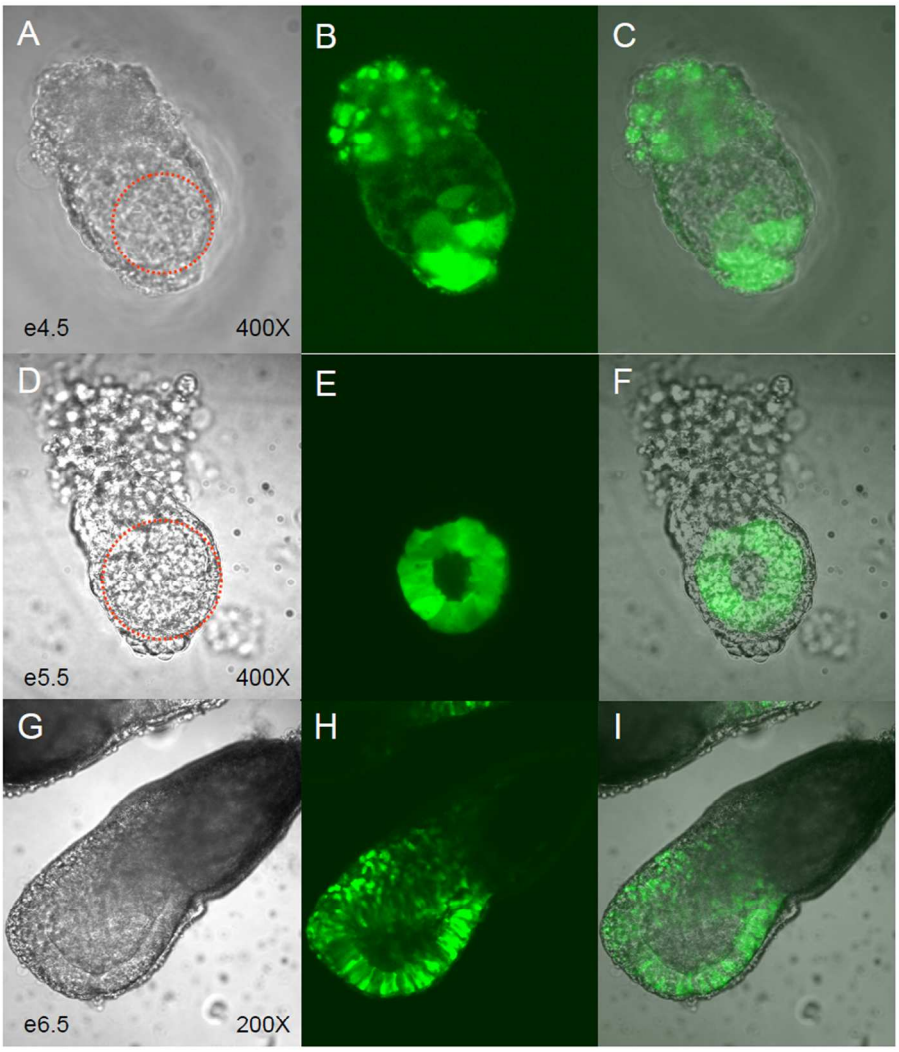


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Figure 4

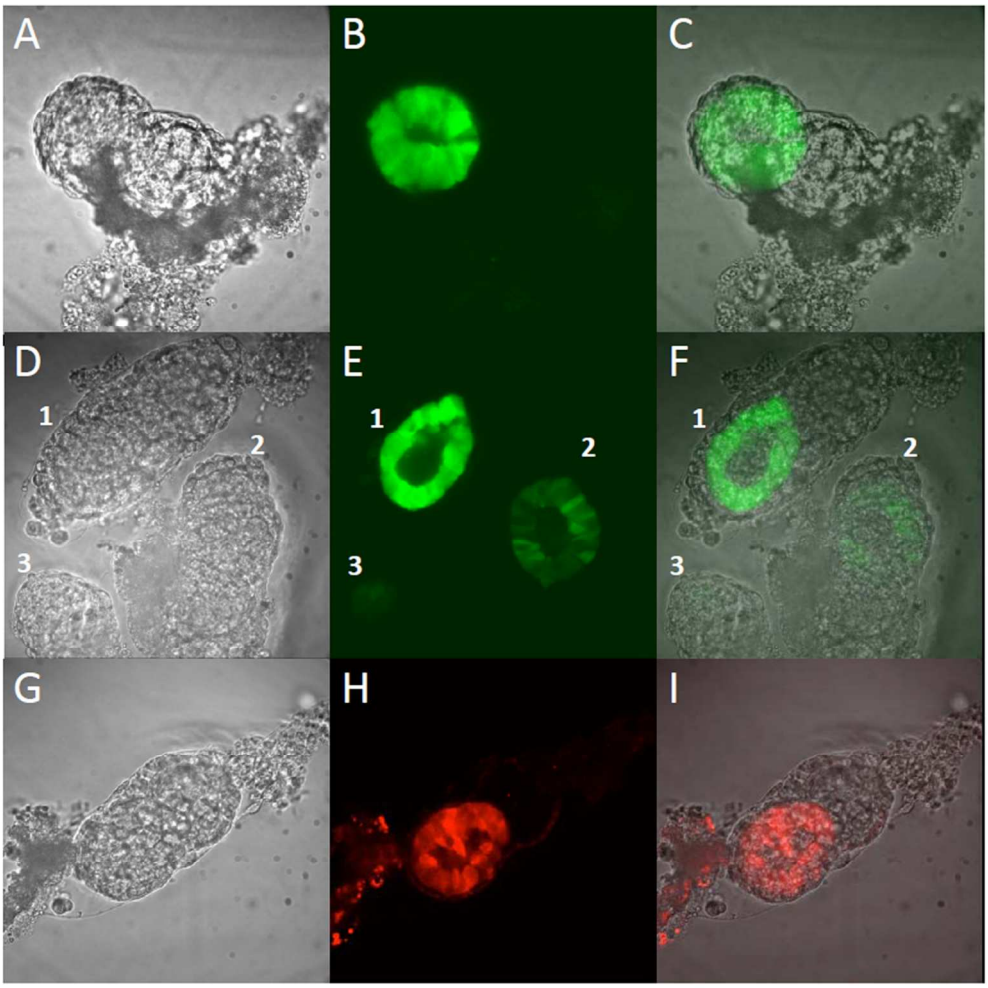


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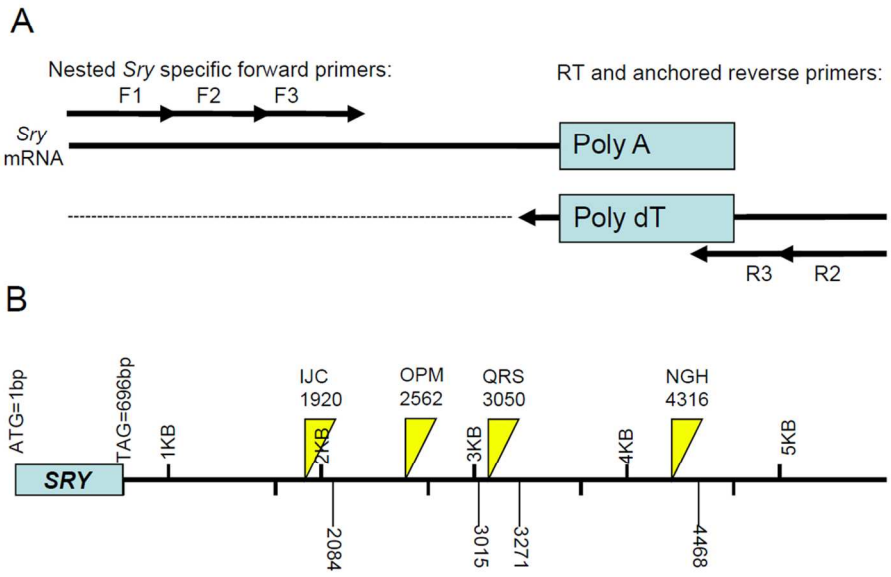
Figure 5




C

| ATG(1) | Tissues | Poly-adenylation site |
|---|---|---|
| 2084 | e4.5<br>Testes | AAAACATACCAAACTTAATACTGT-(polyA) |
| 3015 | e4.5<br>Genital ridge<br>Testes | AAACAATTAAACTACTGTTCTACATGGTCTT-(polyA) |
| 3271 | e4.5<br>Genital ridge<br>Testes | TAACACAAATGAATACAAATATATCCATCCC-(polyA) |
| 4468 | Testes | AAATGCCATTAATAAAGGATTTCTAAAACATT-(polyA) |

114x143mm (300 x 300 DPI)

Figure 6

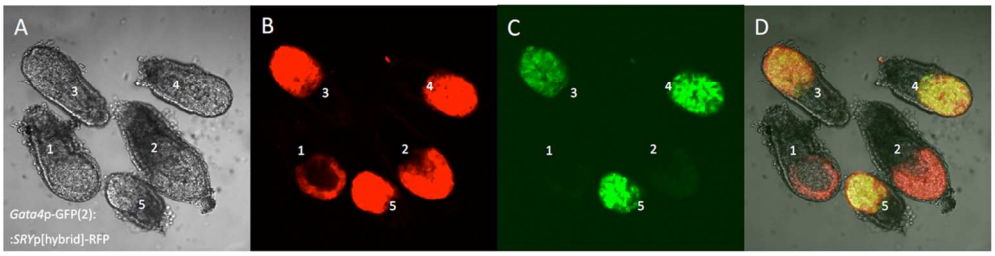



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Figure 7

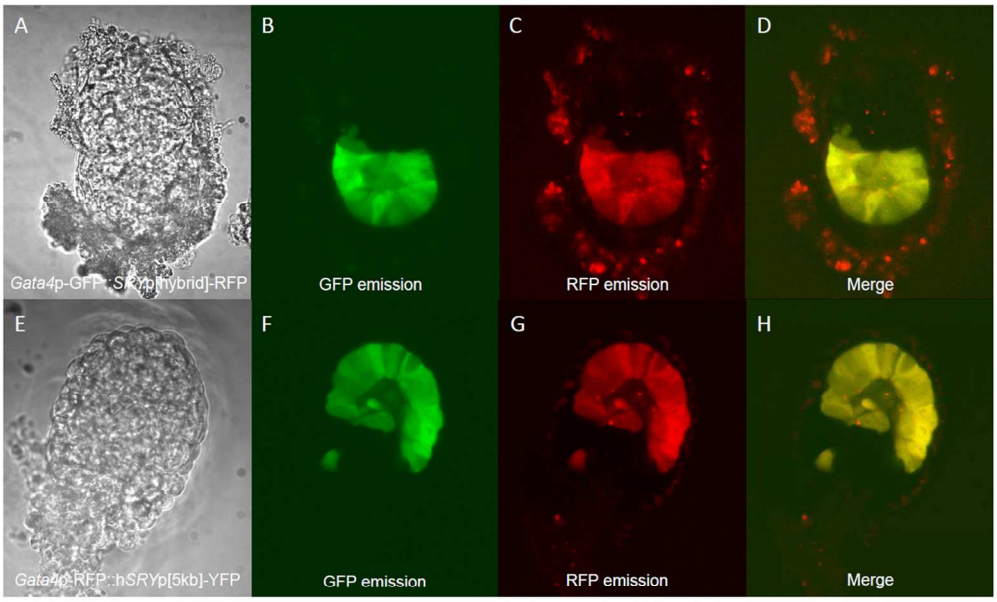


Figure 7
114x87mm (300 x 300 DPI)

Figure 8

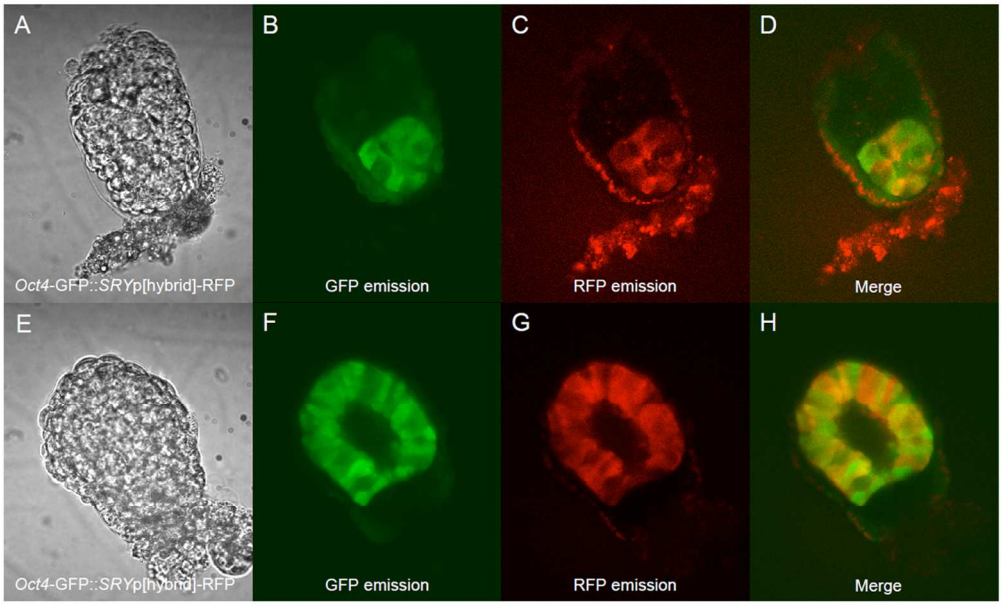


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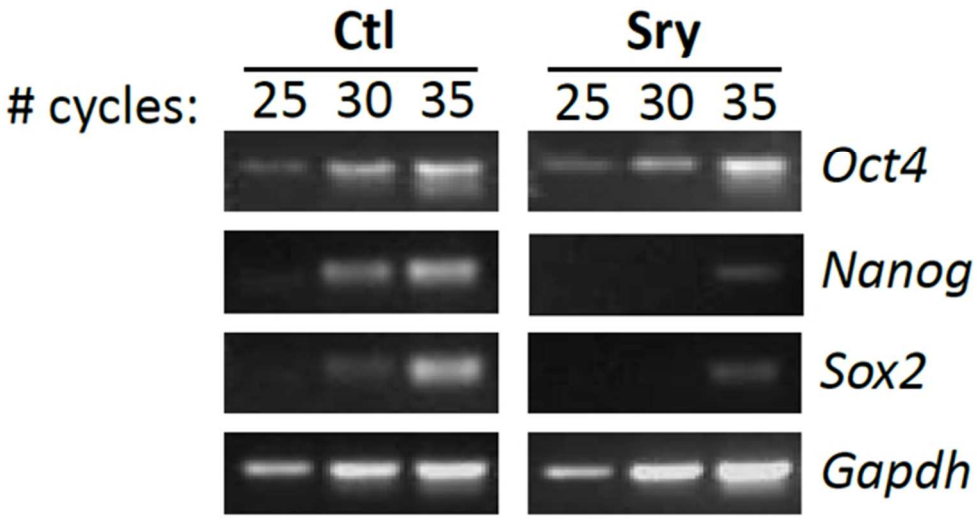
Figure 9

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