Rattling the core of a well-established paradigm: is retinoic acid really necessary for meiosis entry?

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ABSTRACT

Independent studies from recent years have yielded a wealth of evidence supporting the model that retinoic acid (RA) triggers the expression of Stimulated by retinoic acid 8 (Stra8) gene in germ cells thereby inducing their entry into meiosis *(1, 2)*. Kumar et al.'s recent work published in 2011, however, boldly challenges this model, proposing that entry into meiosis, in fact, takes place independently of endogenous RA *(3)*. This controversial claim has attracted intense interest in the field of developmental biology and has prompted several follow-up studies. One of these studies has proposed two homeobox genes, Msx1 and Msx2, to also be necessary for female meiosis initiation, and that their actions are likely mediated by the expression of Stra8 *(4)*.

KEYWORDS

Retinoic acid- the proposed extrinsic inducer of Stra8 in the current model for meiosis entry
Meiosis entry- a critical, sex-specific process in spermatogenesis and oogenesis
Stra8- the unequivocal regulating factor of meiosis entry in male and female germ cells
Cyp26b1- a male-specific, RA-degrading enzyme that inhibits meiosis entry in the testis
Msx1, Msx2- two homeobox genes that have been recently proposed to regulate meiosis entry

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Received: 16 January, 2012
Revised: 2 March, 2012

THE BIG QUESTION

Germ cells enter meiosis at dramatically different times between the sexes: female germ cells enter at around 13 days post-coitum (dpc), but male germ cells do not enter until postnatally, at around 7-10 days post-partum (dpp) *(5)*. This observation frames the central question that many researchers are trying to answer: what determines the sex-specific difference in the timing of meiosis entry?

THE CURRENT PARADIGM

Previous gene knockout studies have firmly established Stra8 as the unequivocal regulator of meiotic program initiation in both spermatogenesis and oogenesis. Indeed, male germ cells from pure C57BL/6 mice depleted of Stra8 gene function did not progress beyond the preleptotene stage *(6)*. Additionally, while germ cells from mice of mixed genetic background were able to complete meiotic replication and enter meiosis, they failed to proceed past the prophase stage *(6)*. Other studies that followed shortly after have confirmed this finding *(7-10)*. In particular, the work of Menke et al. has determined a strong correlation between the wave of anterior-posterior expression of Stra8 and the wave of anterior-poster expression of bona fide meiotic mark-

ers, such as synaptonemal complex protein 3 (Scp3) and Dmc1 [DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)] *(10)*.

Previous studies have suggested that retinoic acid (RA), a vitamin A derivative, is the extrinsic inducer of Stra8 and is thus necessary for regulating the induction of meiosis *(11)*. The very gene designation Stra8 is, in fact, an acronym for "stimulated by retinoic acid." Thus far, RA and its underlying mechanisms of function have been well studied and documented. During embryonic development, RA commonly functions in a paracrine manner. Its local level is finely tuned by the balance between its tissue-specific synthesis by the retinaldehyde dehydrogenases (Radlh1, Radlh2, Radlh3) and its oxidative degradation by the cytochrome P450 enzymes (Cyp26a1, Cyp26b1, Cyp26c1) *(12)*. Once generated, RAs then travel to RA-target cells where they directly regulate genes by binding to two families of nuclear receptors—RA receptors (RARs) and retinoid X receptors (RXRs)—bound to the RA response elements (RAREs) in the regulatory regions of their targeted genes *(12)*. This general model applies differently to different organisms. In mice, evidence suggests that the expression of Raldh2 in the mesonephros serves as the main paracrine source of RA for the adjacent gonad, which does not express Raldh2 *(13)*.

Several areas of study lend support to the necessity of RA in meiosis entry. Two in particular are relevant to this discussion. First, the exogenous treatment of RA is sufficient to stimulate Stra8 expression in embryonal carcinoma cells and embryonic stem cells in culture *(14)*. Second, which was demonstrated more recently, exogenous RA can also stimulate germ cells in the embryonic testes to begin expressing Scp3, Dmc1, and and member X (yH2afx) of the H2A histone family, and undertake meiosis-specific morphology *(11, 13, 15, 16)*.

Besides Stra8 and RA, the P450 enzyme Cyp26b1, normally expressed in Sertoli cells of fetal testis, is the third ingredient in the cocktail of factors essential for regulating entry into meiosis. Importantly, Cyp26b1 has been shown to act as a crucial masculinizing factor that delays meiosis entry in male mice *(10, 13, 17, 18)*. When resident germ cells in male testis were treated with ketoconazole, a potent but non-specific cytochrome p450 inhibitor, expressions of Stra8, Scp3, and Dmc1 were upregulated and these cells proceeded to develop meiosis-specific morphology *(11, 13)*. When these cells were treated simultaneously with ketoconazole and RAR panantagonist BMS-204493, however, meiotic induction ceased to take place. This suggests that ketoconazole, although a non-specific inhibitor of P450 enzymes, acts specifically through the RARs to induce meiosis. This finding lends support to previous studies, which postulated that Cyp26b1 acts to degrade RA. Other tests, including those involving a more specific inhibitor of Cyp26 enzymatic activity, R115866, as well as its treatment in combination with the RAR panantagonist, produced parallel results *(11)*.

Together, this large body of studies generated the most recent and widely-accepted model to explain the sex-specific timing of meiosis entry: RA synthesized in the mesonephros serves as an extrinsic inducer of Stra8 and hence meiosis entry in the adjacent gonad, unless degraded by Cyp26b1, as in the case of fetal testis.

THE PERCEIVED KNOWLEDGE GAPS

Despite the wealth of evidence in support of the current model, Kumar et al. remained skeptical *(3)*. In their discussion, they identify several weak links common to the recent studies. They point out that in the studies involving exogenous RA treatment, including that which led to the first identification of Stra8 as an RA-responsive gene, 'supra-physiological' concentrations were used, which clearly could not be reflective of the endogenous reality. They also raise concerns over findings from studies using the RAR antagonists, stating that they are known to exert non-specific effects on receptors other than the RARs *(19)*. Additionally, they suggest that commonly used RALDH inhibitors for studying meiosis entry, including the disulfram compounds Win18,446 and citral, are known to also inhibit other aldehyde dehydrogenases that have no relevance to retinoid metabolism *(20)*. This means that such RALDH inhibitors are likely to produce artifacts and misinterpretations. Kumar et al., doubtful of the validity of the current model, set out to investigate further *(3)*.

THE RATTLING DISCOVERY

Kumar et al. *(3)* set out to determine whether endogenous RA is indeed, as proposed by the current model, necessary for the induction of meiosis entry. To investigate this link, they looked at meiosis in fetal ovary null for either Raldh2 alone or in combination with Raldh3. Surprisingly, despite the absence of these two major RA synthesizing enzymes, Stra8 was still expressed and bona fide meiosis markers Scp3 and ɣH2AX protein were still detectable. This finding implies that meiosis had occurred normally. But was RA really absent in the fetal ovaries of these mutant mice? To test this, they used a transgenic RARE-LacZ mouse line to report the RA activity, and assuredly, they did not detect its level in the mesonephros or the developing ovary of the mutant mouse background. Further tests also confirmed that the RARE used in the RARE-LacZ reporter construct was indeed sensitive enough, at least more sensitive than the putative Stra RARE found in the endogenous system, to detect the endogenous level of RA.

Inhibition of Cyp26b1 in fetal mutant testes, which lack a source of putative Stra8-inducing RA from the adjacent mesonephros, still led to the induction of Stra8 expression. However, when the mesonephros was removed from the testes, Stra8 expression was not induced.

All these findings led Kumar et al. to two main conclusions: 1) Stra8 expression remains unaffected in the absence of RA in the fetal ovary and is therefore not required for the induction of meiosis during ovary development; 2) some extrinsic signal, other than RA, is required from the mesonephros to induce meiosis, and the role of Cyp26b1 is not to degrade endogenous RA, as proposed by the current model, but rather to block that non-retinoic, meiosis-inducing signal *(3)*.

Kumar et al.'s findings have flared up a whirlwind of contention in the field of developmental biology. While some groups hail the new possibilities, other groups vehemently protect the integrity of the current model.

THE PROTECTORS *(21)*

Griswold et al., for one, rest in favour of the current model and have offered alternative interpretations to Kumar et al.'s observations. First, in regards to the observation that meiosis could be induced in the absence of RA, they argued that there remains the possibility that RA persisted in Kumar et al.'s experimental system. Even though the mutant mice were null for the major RA producing enzymes, Raldh2 and Raldh3, Raldh1 was unaccounted for. Raldh1 is also capable of synthesizing RA and it could have been feeding out low amounts of RA, which in turn drove the meiosis in Kumar et al.'s system.

But if RA was present in the system, why was its activity undetected by the RARE-LacZ reporter construct? Kumar et al. clearly demonstrated that the technique is sensitive enough to detect the levels of RA required to induce Stra8 activity. To address this, Griswold et al. pointed to a recent study investigating the reliability of RARE-LacZ construct. This paper concluded that, although a powerful technique, RARE-LacZ remains prone to losing responder activity, which could lead to a failure in detecting RA *(22)*.

Griswold et al. also questioned the reliability of Kumar's experimental system setup itself. To overcome the embryonic lethality associated with RA deficiency of the mutant mice null for the retinaldehyde dehydrogenases, Kumar et al. administered to these mice exogenous RA up to 9dpc before clearing it away. Griswold et al. argue that adding exogenous RA necessarily complicated their experimental system and gave rise to unwanted possibilities that were unaccounted for in Kumar et al.'s study, such as delayed effects of early RA exposure that later trigger meiosis. Given that no quantitative evidence had been presented to prove that RA was indeed completely cleared from the gonads post-administration, the possibility that residual amounts of RA persisted at low levels in these systems cannot be discounted. Griswold et al. further contend that given the lack of evidence that Cyp26b1 can degrade non-retinoid compounds, the simplest explanation for Kumar et al.'s findings is that their observations are attributed to RA.

THE HAILERS *(4)*

On the other end of the spectrum, other researchers are pushing to elucidate the implications of Kumar et al.'s findings. In particular, Livera et al.'s most recent publication proposed two new essential factors that regulate meiosis initiation: homeobox proteins Msx1 and Msx2. Until now, homeobox proteins have not been implicated in meiosis entry control. Msx homeobox proteins are important for the correct development of many organs, including the limbs, teeth, and neural crest *(23)*.

Using the Msx1ERT2cre mice with a ROSAmT/mG reporter system, Livera et al. confirmed that Msx1 protein is expressed in germ cells in the fetal ovary at 13 dpc, suggesting a correlation between Msx gene expressions and female fetal meiosis initiation. Furthermore, the double knockout (dKO) of Msx1 and Msx2 prevented most germ cells in the female ovary from initiating meiosis. Msx genes appear to function specifically in female meiosis, having no detectable level of activity in males when meiosis begins in the post-natal testis. Other studies involving morphological observations of female germ cells that did not undergo meiosis demonstrated that Msx is likely required solely for the initiation step of meiosis, but not for its progression through prophase I. This was demonstrated by a subset of cells which had successfully initiated meiosis at 14.5 dpc in the dKO ovaries, and have progressed normally through meiosis to the diplotene stage.

More relevant to this discussion is Livera et al.'s observation that Stra8 could be a target of Msx proteins, as overexpression of Msx1 increased Stra8 expression and Msx1 was found to bind the Stra8 promoter. The time period during which Msx proteins are needed to act on Stra8 and the fact that other co-factors may be involved in the process, however, are questions that still need to be answered.

WRAPPING UP

Evidently, studies into the role of homeobox genes in regulating meiosis initiation is at a premature stage, to say the least, and more questions were posed than were answered by Livera et al.'s work. Nevertheless, this report work presents a promising outlook on a new field of study waiting to emerge.

Furthermore, on the question of whether RA is indeed required for meiosis, the two pivotal works by Kumar et al. and Livera et al., along with other recent studies, certainly demonstrate that thus far, we have but an incomplete picture. Beyond the relationship between RA, Stra8 and meiosis initiation, many other factors and modulators are also involved, including DAZL, FGF9, and DMRT1, which have been recently identified. In the end, this is perhaps entirely expected of a critical developmental process that would ultimately result in the formation of the complex organisms that mammals are. Whatever the case may be, one thing is for certain: the study of regulation of meiosis entry has just begun, and these recent breakthroughs will pave the way for many more exciting discoveries to come.

ACKNOWLEDGEMENTS

I wish to thank Dr. Hugh Clarke and Dr. David Dankort for guiding me in writing this paper.

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