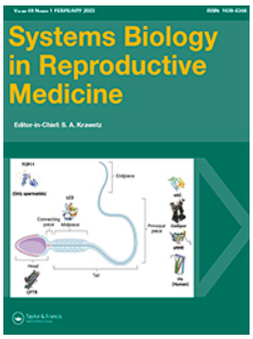

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REVIEW AND HYPOTHESIS

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Trophectoderm non-coding RNAs reflect the higher metabolic and more invasive properties of young maternal age blastocysts

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ABSTRACT

Increasing female age is accompanied by a corresponding fall in her fertility. This decline is influenced by a variety of factors over an individual's life course including background genetics, local environment and diet. Studying both coding and non-coding RNAs of the embryo could aid our understanding of the causes and/or effects of the physiological processes accompanying the decline including the differential expression of sub-cellular biomarkers indicative of various diseases. The current study is a post-hoc analysis of the expression of trophectoderm RNA data derived from a previous high throughput study. Its main aim is to determine the characteristics and potential functionalities that characterize long non-coding RNAs. As reported previously, a maternal age-related component is potentially implicated in implantation success. Trophectoderm samples representing the full range of maternal reproductive ages were considered in relation to embryonic implantation potential, trophectoderm transcriptome dynamics and reproductive maternal age. The long non-coding RNA (lncRNA) biomarkers identified here are consistent with the activities of embryo-endometrial crosstalk, developmental competency and implantation and share common characteristics with markers of neoplasia/cancer invasion. Corresponding genes for expressed lncRNAs were more active in the blastocysts of younger women are associated with metabolic pathways including cholesterol biosynthesis and steroidogenesis.

Abbreviations: YMA: Young Maternal Age; IMA: Intermediate Maternal Age; AMA: Advanced Maternal Age; Rba: Reproductive Biological Age; lncRNA: long non-coding RNA; ART: Assisted Reproductive Technology; WHO: World Health Organization; ASRM: American Society for Reproductive Medicine; PGT-A: Preimplantation Genetics Testing for Aneuploidy; RBP: RNA binding protein; CPM: Copies-Per-Million mapped reads; FDR: False Discovery Rate; AMH: Anti-Müllerian Hormone; miRNA: microRNA; NMD: nonsense-mediated decay

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Gene expression; RNA sequencing; trophectoderm; long non-coding RNA; maternal age; blastocyst

Introduction

Blastocyst implantation is a highly complex process relying on close coordination between the attaching embryo and the receptive endometrium, with approximately 50% of good quality blastocysts failing to implant (Craciunas et al. 2019). Human blastocyst formation occurs while the developing zygote is transiting through the fallopian tube, normally reaching the uterine cavity on the 5th day after successful fertilization (Norwitz et al. 2001; Figure 1). Embryo-endometrial communication supported by various cell surface and secreted factors is thought to take place at the blastocyst stage, preparing the embryo for initial adhesion and attachment (Simón et al. 2000; Ashary et al. 2018). Implantation itself follows the firm attachment of the embryo to the endometrium's luminal epithelium.

Infertility, causes and reproductive age

According to the World Health Organization (WHO) data, approximately 48 million couples and 186 million individuals are considered subfertile or infertile globally (Rutstein and Shah 2004; Boivin et al. 2007;

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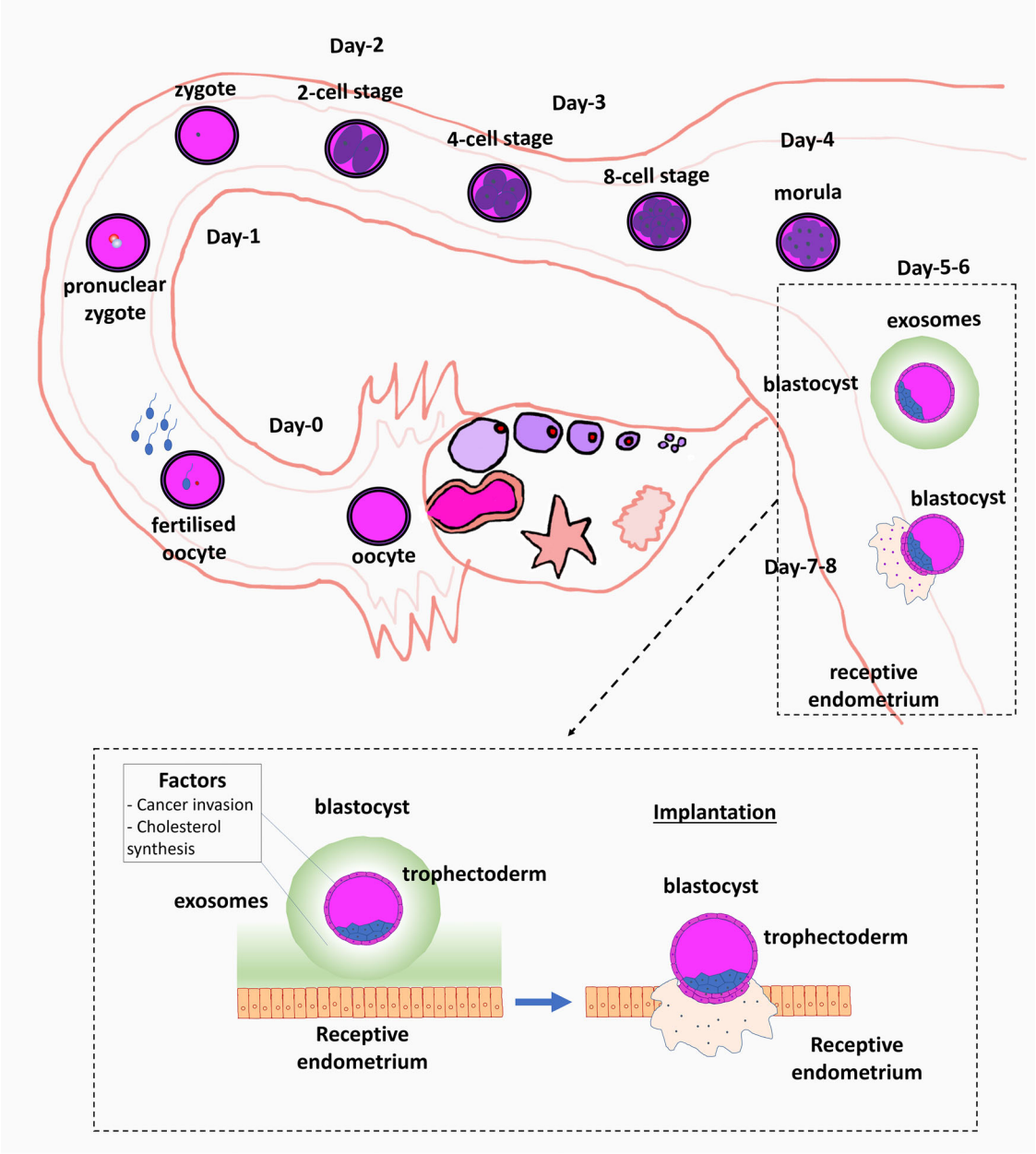

Figure 1. Illustration of the female reproductive system. The image shows the processes of ovulation, fertilization, the different stages of embryonic development and implantation (mid-right dash rectangle). The trophectoderm and/or exosomal factors (lncRNAs) that could participate in the implantation process are also illustrated (bottom dash rectangle).

Mascarenhas et al. 2012). Unexplained/idiopathic infertility accounts for up to 25–30% of this burden (Medicine PCotASfR 2006b) and by investigating different aspects of embryo development, including their gene expression patterns (RNA/molecular biomarkers), it should be possible to improve implantation/pregnancy rates (Collins and Van Steirteghem 2004; Saravelos and Li 2012; Gelbaya et al. 2014; Sadeghi 2015).

Ageing is responsible for reduced fertility in women (Nelson and Lawlor 2011; Schmidt et al. 2012) and even with the use of assisted reproductive technology (ART), when euploid embryos derived from women over the age of 35 are transferred into the uterus compared with the transfer of embryos from younger women, live birth rates are reduced (Scott et al. 2012). Moreover, implantation and pregnancy rates continue falling among women of 40 years and above (Hull et al. 1996; Medicine PCotASfR 2006a). While the ageing endometrial environment may play a part in the age related decline in female fertility, the relatively high pregnancy rates achieved in older women undergoing treatment by egg donation from younger women, indicates that a major cause of the age related decline in fertility is the declining quality

of oocytes and embryos of older women (Wang YA et al. 2012). Hence, maternal age is a variable that could help reveal molecular biomarkers and processes that support implantation/pregnancy among younger mothers and could help to improve treatment success rates among reproductively older women.

The American Society for Reproductive Medicine (ASRM) guidelines indicate that the male (as the sole or contributing factor) causes approximately 40% of all cases of infertility, while female infertility as sole factor, accounts for another 40% (Kumar and Singh 2015; Walker and Tobler 2020), along with various other uncharacterized/undetermined parental factors (Wu H et al. 2017; Colaco and Sakkas 2018). The mammalian blastocyst consists of trophectoderm cells (outer layer) that gives rise to the extra-embryonic tissues including the placenta and the inner cell mass (internal cells or ICM) that ultimately gives rise to the fetus. Hence, the trophectoderm is the first embryonic tissue to communicate directly with the endometrium during a narrow implantation window (Figure 1). Implantation and pregnancy failure may be affected by various factors including a failure of communication and synchronization between the blastocyst and endometrium (Achache and Revel 2006; Margalioth et al. 2006) caused by uterine anomalies (Taylor and Gomel 2008) and/or embryonic factors such as chromosomal aneuploidies (Harper 2018; Cimadomo et al. 2020) and abnormal gene expression (McCallie et al. 2019; Ntostis et al. 2019; Abu-Halima et al. 2020; Ntostis et al. 2021). These deficiencies can be mitigated in part, by ensuring only transfer of euploid blastocyst where implantation rates from 50%-80% are observed (Saravelos and Li 2012).

Approaches used in assisted reproductive technology

Contemporary clinical ART practice principally relies on two characteristics of couples undergoing ART treatment. The first relies on whether the male has sufficient numbers of normal spermatozoa to support natural oocyte fertilization, which can, for example, determine whether Intra-Cytoplasmic Sperm Injection (ICSI) is required or not. The second characteristic where visual indicators of developing embryo quality (scored according to morphological criteria) are used to select those considered the most likely to implant, depends on both partners. Morphological criteria are frequently combined and scored subjectively to assess embryo quality. The elements contributing to the score include successful trophectoderm formation, inner cell mass development, expansion and hatching, all of which are aimed at improving implantation and pregnancy success rates (Gardner et al. 2000; Gardner and Balaban 2016; Figure 1). The embryo quality score can be combined with Preimplantation Genetic Testing for Aneuploidy (PGT-A) to prevent the transfer of aneuploid blastocysts (Cimadomo et al. 2020; Handyside 2020). These main ART approaches are sometimes complemented by additional tests, including detection of the presence of antisperm antibodies (Sundaram et al. 2019), the level of (sperm) DNA fragmentation (Agarwal et al. 2020) and the levels of anti-Müllerian hormone (AMH) (Oh et al. 2019). Other tests consider endocrine status (Zhao et al. 2020), which combined with genetic markers (Jodar et al. 2015; Ntostis et al. 2017; Estill et al. 2019), morphokinetics and metabolomics (Meseguer et al. 2011; Hardarson T. et al. 2012; Herrero and Meseguer 2013) could help assess the fertility status of the couple and the quality of their embryo(s) selected for transfer. The newly burgeoning 'omics' era offers additional new methodologies that could help raise the corresponding implantation success rates (Hardarson et al. 2012; Gardner and Balaban 2016).

PGT-A can detect aneuploidies that arise more frequently among the embryos of older women (Cimadomo et al. 2020); the benefits of this approach, however, have recently been challenged (Harper 2018). The method is best suited to cases where parents have known genetic anomalies or when women of particularly advanced reproductive age undergo ART treatment. PGT-A is less effective among younger couples where aneuploidy rates are much lower. From the maternal perspective, endometrial transcriptomic signatures that define the best implantation window (high endometrial receptivity) can also be considered (Díaz-Gimeno et al. 2011). This approach, however, does not consider additional embryonic markers that may be affecting implantation dynamics of embryo(s) following their intra-uterine transfer.

Considering that approximately a third of infertility in couples is idiopathic in nature (Collins and Van Steirteghem 2004; Saravelos and Li 2012; Gelbaya et al. 2014; Sadeghi 2015), certain characteristics/parameters of their blastocysts may be responsible for implantation success (or failure). Embryonic gene expression profiles for example, are gradually altered as maternal age rises and these alterations could explain aspects of idiopathic infertility associated with increasing maternal age. The RNAs associated with the less fertile/ageing population could mirror abnormal transcriptome profiles following fertilization and

subsequently abnormal embryonic development. Epigenetic factors, such as aberrant methylation patterns and histone modifications that result in shaping the trophectoderm transcriptome and associated implantation success or failure, may also be important (Scott et al. 2012; Harton et al. 2013; Lee et al. 2015). These molecular biomarker could be used to 'flag' the selection of the most competent blastocyst(s) for embryo transfer, improving fertility rates (Saravelos and Li 2012; Gelbaya et al. 2014).

In this regard, additional markers associated with implantation and pregnancy outcomes include various embryonic/trophectoderm RNAs (short/long and coding/non-coding) that reflect the health of the embryo and its competence to implant. Trophectoderm coding RNAs (Ntostis et al. 2019; Ntostis et al. 2021) along with other embryonic small non-coding RNAs (Cuman et al. 2015) may help to indicate disease/fertility status and the implantation/pregnancy potential of the corresponding blastocysts. These biomarkers may be useful regardless of whether the embryo sampling method used is invasive (i.e., trophectoderm biopsy) or non-invasive (analysis of spent culture media). The latter relies on secreted/extracellular RNA biomarkers reflecting, for example, aspects of successful human embryo implantation (Paul et al. 2019; Ntostis et al. 2021; Zmuidinaite et al. 2021).

The roles of long non-coding RNAs

The interplay between non-coding RNAs and double stranded DNA in mediating gene expression (Mondal et al. 2015; O'Leary et al. 2015) or silencing (Martianov et al. 2007; Schmitz et al. 2010) is now more widely accepted. The position of a long non-coding RNA (lncRNA) with respect to its neighbouring genes could play an important role in their regulatory function. Moreover, by regulating neighbouring loci and/or by generating a chromatin state that affects the expression of genes lying nearby (Gil and Ulitsky 2020), lncRNAs play an important role in *cis* regulatory elements (Luo et al. 2016). LncRNAs can interfere with the transcription machinery, promoting gene silencing by altering the accessibility to and recruitment of transcription factors including RNA polymerase II, or by altering/inducing specific histone modifications affecting gene expression (Thebault et al. 2011; Latos et al. 2012; Stojic et al. 2016; Rom et al. 2019).

In particular, lncRNAs can modulate chromatin functions (Widom 1998; Mercer and Mattick 2013), interfere with various signalling pathways and modulate cytoplasmic mRNA stability and translation, making them potentially effective biomarkers (Statello et al. 2021). LncRNAs also interact with RNA binding proteins (RBPs) and ribosomes in the cytoplasm (Carlevaro-Fita et al. 2016) and with mitochondria (Mercer et al. 2011) or other organelles including exosomes (Li S et al. 2018; Statello et al. 2018). Exosomes package both coding and non-coding RNAs, which are functional when transferred to recipient cells (Valadi et al. 2007) and are involved in various biological functions, including the transfer of genetic material, signal transduction and cellular communication. Exosomal lncRNAs are involved in the regulation of gene expression, histone modifications, alternative RNA splicing, cellular reprogramming, epigenetic regulation and the genomic stability of recipient cells that by modifying gene expression, could potentially influence the development of cancer phenotypes (Fatima and Nawaz 2017). They play important roles in modulating the immune system, along with their involvement in cancer and other inflammatory diseases (Turpin et al. 2016; Wang WM et al. 2019).

Other functionalities involve the formation of a triple helix structure upstream of gene enhancers, that help recruit chromatin modifiers and promote enhancer *RNA* (eRNA) transcription (Postepska-Igielska et al. 2015; Blank-Giwojna et al. 2019). LncRNAs can regulate gene expression at the post-transcriptional, translational and post-translational level. They bear complementary regions that compete with microRNAs (miRNAs) targeting specific mRNAs, expression of which ultimately relies on their relative proportions (Bosson et al. 2014; Denzler et al. 2014; Grelet et al. 2017). They can also act as scaffolds supporting the generation of ribonucleoprotein complexes and as a guide that could bring these complexes to specific genome locations (Bouckenheimer et al. 2016). Finally, they can bind to proteins preventing interactions with their targets, providing in this regard a post-translational regulatory mechanism (Karreth et al. 2014).

Various lncRNAs are expressed during human early embryonic development (Yan et al. 2013), supporting cellular development, pluripotency, differentiation (Loewer et al. 2010; Guttman et al. 2011; Rosa and Brivanlou 2013) and the regulation of gene expression (Fatica and Bozzoni 2014), including the regulation of *HOX* gene regions (Rinn et al. 2007). LncRNAs are responsible for genomic imprinting, with aberrations in imprinted regions associated with various disorders (Autuoro et al. 2014; Azzi et al. 2014). A DNA methylation signature of lncRNAs has been suggested for

human pre-implantation embryos (Li J et al. 2017). LncRNAs are also required for X-chromosome inactivation (Penny et al. 1996). The absence of lncRNAs on the other hand, has been associated among others with the induction of oxidative stress and apoptosis (Zhang A et al. 2013).

LncRNAs, which are normally expressed in the pre-implantation embryo are aberrantly expressed in various cancers (Ben-Porath et al. 2008; Schoenhals et al. 2009) and are involved among others in cancer development/progression, including cell proliferation/survival, metabolism and participation in the formation of the tumor microenvironment (Batista and Chang 2013; Yuan et al. 2014). Trophectoderm lncRNAs are likely to be involved in embryonic implantation, which is akin to invasive and inflammatory processes (Griffith et al. 2017) typical of carcinogenesis. As presented below, the extension of our transcriptomic studies on the effects of aging on implantation revealed lncRNAs. This has opened a pathway to a mechanism highlighting their potential clinical application discussed below.

Current approach

To investigate trophectoderm biomarkers, we first compared transcriptomic profiles of blastocysts that implanted successfully with those that failed to implant (Ntostis et al. 2019). The human trophectoderm sample size was then further increased ($N = 32$) and the generated data used in a different experimental setting to identify the trophectoderm transcriptome (coding transcripts) associated with maternal age, implantation success and potentially with embryo-endometrial communication. Euploid trophectoderm samples were clustered based on maternal age (chronological and/or biological) (Ntostis et al. 2021), with samples also assessed, as previously reported, in the context of endometrial receptivity (Hu et al. 2014; Altmäe et al. 2017). This work revealed factors of potentially great importance in embryo-endometrial communication and ultimately implantation (Ntostis et al. 2021). The hypothesis that long non-coding RNA affects implantation success, arose by re-examining the RNA sequencing data from these studies in the context of maternal ageing and embryo implantation (Ntostis et al. 2021). Here, we extend the reach of the study to investigate the characteristics of non-coding poly(A) containing RNAs as a potential tool for predicting successful implantation following day 5 blastocyst uterine transfer and address the question of whether non-coding RNAs can be used as molecular biomarkers to predict blastocysts with the highest potential to implant.

Results and discussion

Exploring trophectoderm gene expression patterns

Maternal age-related epigenetic and transcriptomic factors could affect the embryonic development and implantation (McCallie et al. 2019; Ntostis et al. 2019; Ntostis et al. 2021). In this analysis, RNA-Seq data from a total of 32 euploid samples were considered. Ten samples were derived from 4 women aged up to 30 years (Young Young Maternal Age; YMA), 16 were from 8 women aged between 30 and 39 years (Intermediate Maternal Age; IMA) and 6 were from 3 women aged at least 40 years (Advanced Maternal Age; AMA) with an average maternal age per blastocyst of 24.4 ± 2.0 years, 34.3 ± 2.6 years and 42 ± 1.1 years (mean ± SD) (Figure 2). The blastocysts of a single woman/donor all derived from the same cycle.

Hierarchical clustering analysis on the trophectoderm gene expression including both coding and non-coding transcripts, was used as a guide to define the profiles associated with reproductive biological maternal age (rba). The samples were allocated into 3 groups, namely rba-YMA, rba-IMA and rba-AMA. Transcriptome patterns of the rba-YMA and rba-AMA groups were more distinct from each other compared with the rba-IMA group. Moreover, the YMA, AMA and IMA samples were closely associated with their respective reproductive biological age rba-YMA, rba-AMA and rba-IMA groups, respectively, with the IMA samples having a less clearly defined distribution that classified them into the 3 rba groups (Ntostis et al. 2021; Figure 2). Corresponding paternal ages in the YMA, IMA and AMA groups were 43.6 ± 5.7 years, 40.3 ± 6.5 years, and 41.3 ± 2.5 years, respectively. The aim of this analysis, however, was to further investigate the differentially expressed lncRNAs.

Maternal age, trophectoderm gene expression profiles and implantation success

The current study revealed over 6000 and 3800 long non-coding RNAs when a read CPM of 1 and 5 was employed, respectively, in at least 4 trophectoderm samples. Other studies revealed similar results, with the RNA-Seq analysis performed by Yan and colleagues (2013) revealing over 3400 lncRNAs, when single human pre-implantation embryos and human embryonic stem cells were considered (Yan et al. 2013; Bouckenheimer et al. 2016).

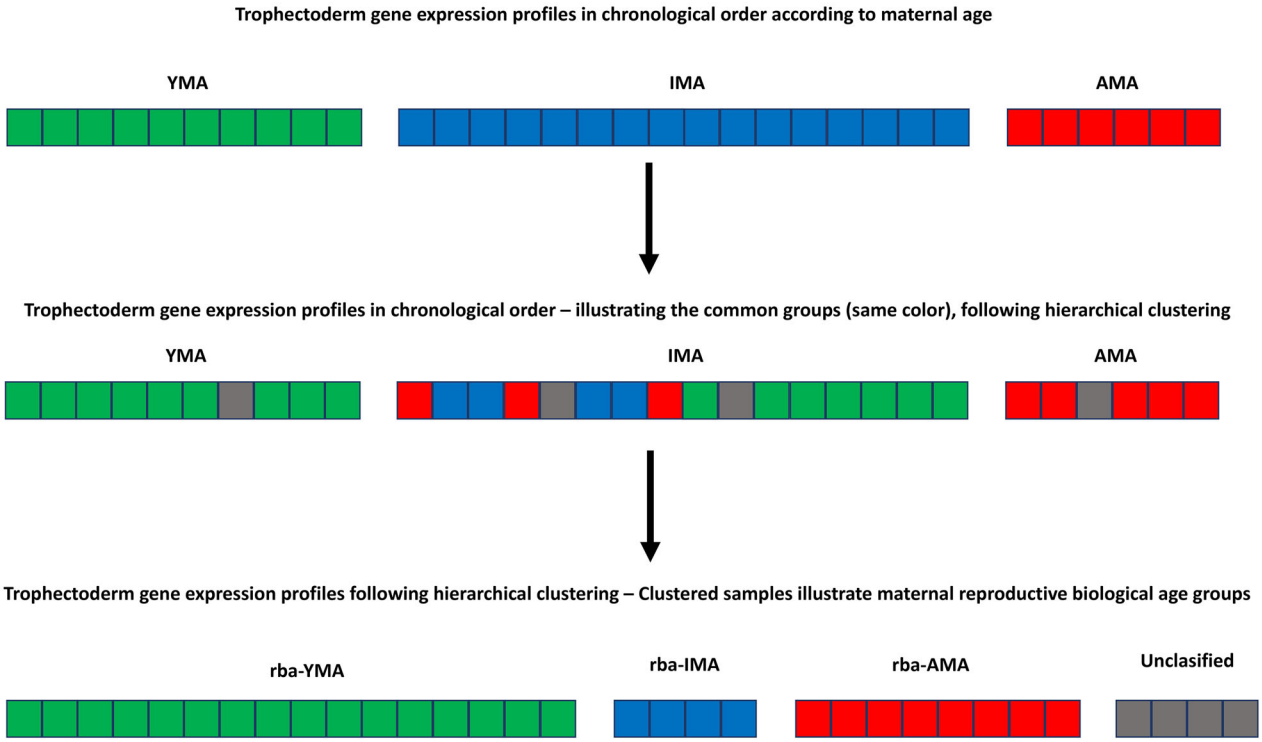

Figure 2. Trophectoderm samples classified according to the blastocyst maternal age or the trophectoderm gene expression profiles. The Young Maternal Age (YMA – green color), Intermediate Maternal Age (IMA – blue color) and Advanced Maternal Age (AMA – red color) samples, correspond to women aged below 30, between 30 and 39 and over 40 years old, respectively. Hierarchical clustering analysis showed consistency in the gene expression of the YMA and AMA groups, that continue clustering in the rba-YMA and rba-AMA groups, respectively. Hierarchical clustering analysis of the gene expression levels of the trophectoderm samples separated them into the 3 reproductive biological age (rba) categories (rba-YMA, rba-IMA and rba-AMA). Grey colour indicates unclassified gene expression patterns that did not match with any of the previous 3 groups.

Differential expression analysis of the YMA, IMA and AMA trophectoderm transcriptomes reported both coding and non-coding transcripts. Hierarchical clustering analysis and classification of the trophectoderm transcriptome in separate groups based on their gene expression patterns, revealed a biological age component that corresponded with implantation success potential, as previously reported (Ntostis et al. 2021) and also confirmed here. The biological maternal age groups rba-YMA, rba-IMA and rba-AMA represent a transcriptome classification where the rba-YMA and rba-AMA groups showed significantly different implantation success rates (Fisher's Exact test; P-value = 0.01), supporting the hypothesis that YMA trophectoderm factors indicate higher likelihoods of implantation success. The current analysis explored the potential functionality of these YMA/rba-YMA and AMA/rba-AMA lncRNAs, their potential roles in the implantation process and the functions that they share in common with tumor development/invasion (Figure 3).

Following differential expression analysis of the trophectoderm transcriptomes from women of various ages, 36 non-coding transcripts were significantly more highly expressed in the YMA/rba-YMA compared with the AMA/rba-AMA groups (Table 1), while 42 non-coding transcripts were more highly expressed in the AMA/rba-AMA compared with the YMA/rba-YMA groups (Table 2). Further investigation of these lncRNA transcripts from the young/successfully implanted blastocysts involved the use of ontological analysis on their potential gene targets, alongside comparisons with various databases and the existing literature, illustrated functionalities indicative of successful implantation (Supplementary Table 1). Blastocysts that derived from mothers of advanced age with reduced implantation potential, showed lncRNA expression profiles characteristic of the aging process (Supplementary Table 2).

Trophectoderm lncRNA characteristics from young maternal age blastocysts

As reported previously (Ntostis et al. 2019; Ntostis et al. 2021) and also confirmed by the current analysis, compared with AMA/rba-AMA blastocysts, the YMA/rba-YMA blastocysts had a higher likelihood of successful endometrial implantation and steroidogenesis-related

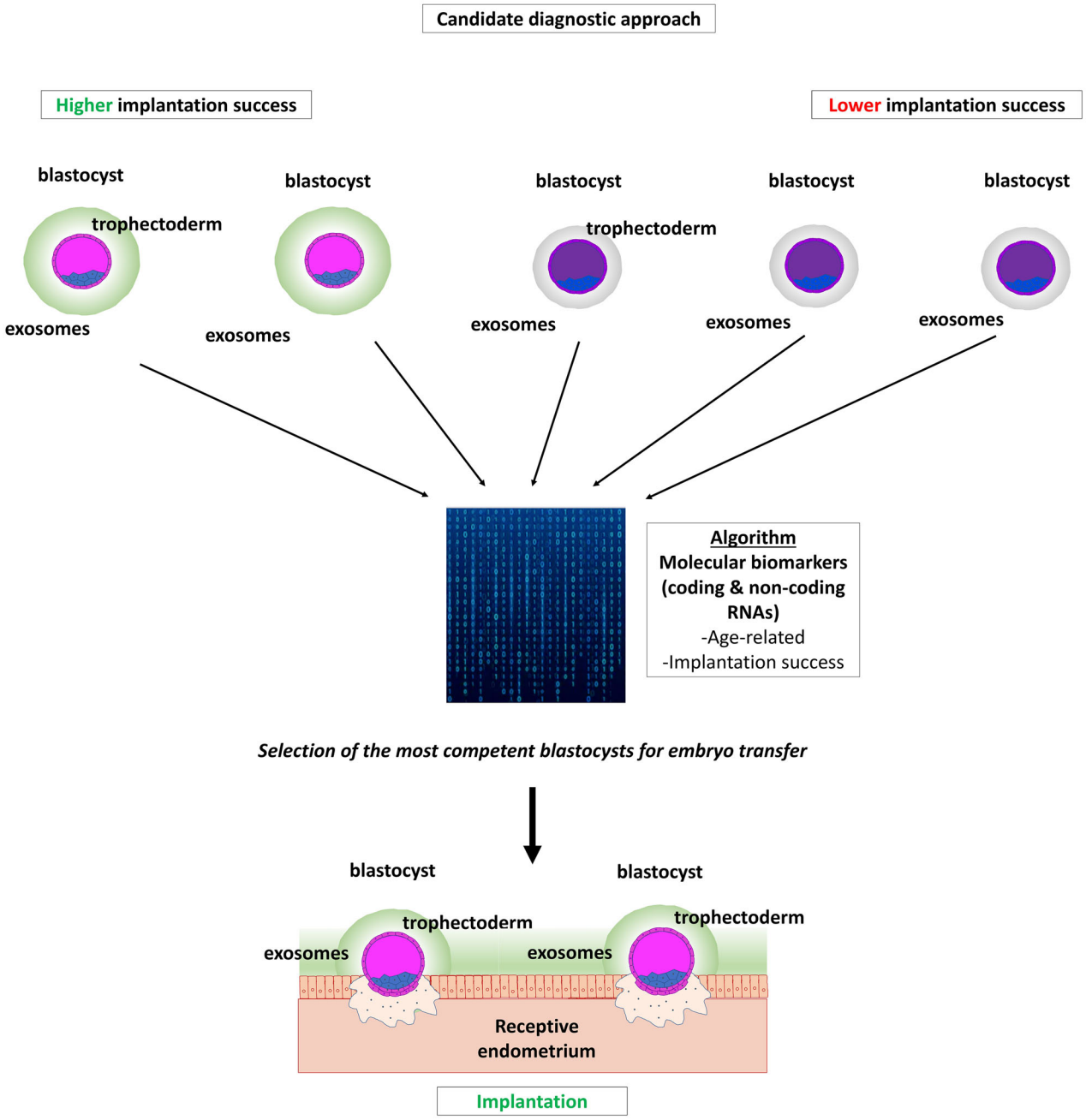

Figure 3. Diagnostic approach that employs the trophectoderm gene expression levels for selecting the most competent embryos for transfer. Five blastocysts from the same couple are shown at the top of the diagram, 2 of which (top left) are more likely to implant into the endometrium and the rest 3 (top right) are more prone to implantation failure. Age- and implantation-associated gene expression data (molecular biomarkers) from all available blastocysts feed the algorithm and can be used for the selection of the blastocysts with the higher implantation potential that will result in implantation success when transferred in the receptive endometrium.

coding transcripts were significantly more highly expressed in the YMA/rba-YMA group, suggesting a potential role for steroidogenesis in early embryo development and/or implantation success. The current study, however, illustrated that differential expression analysis between the YMA/rba-YMA and AMA/rba-AMA transcripts consisted of lncRNAs that could act by modifying the expression of cholesterol- and steroidogenesis-related genes, potentially modulating steroidogenesis (Zhang R et al. 2020; Otsuka et al. 2021). In line with these findings, steroidogenesis also plays an important role in regulating inflammation and the immune system (Chakraborty et al. 2021), potentially affecting embryonic implantation (Van Mourik et al. 2009).

Table 1. Long non-coding RNAs significantly higher expressed in the YMA/rba-YMA blastocysts.

RefSeq ID	Corresponding gene name	logFC	FC	CPM	*p* Value	FDR
NR_144397	*HSD17B1*	3.44	10.83	32.45	2.80E-10	2.30E-07
NR_144402	*LOC108783654/ HSD17B1-AS1*	3.22	9.33	27.28	9.53E-10	6.90E-07
NR_049759	*IFITM3*	3.00	7.99	52.71	7.55E-09	4.51E-06
NR_134300	*IDI1*	1.85	3.59	99.04	1.24E-07	4.73E-05
NR_026598	*NENF*	1.64	3.12	45.57	1.93E-07	6.12E-05
NR_145426	*RUSC1-AS1*	1.66	3.15	37.27	2.36E-07	7.11E-05
NR_117089	*CD24*	3.64	12.49	22.63	4.17E-07	1.06E-04
NR_038118	*MMAB*	2.51	5.71	15.78	6.19E-07	1.39E-04
NR_046298	*SLC25A1*	2.04	4.10	72.50	7.45E-07	1.62E-04
NR_109934	*PCLAF*	2.71	6.55	9.06	1.08E-06	2.12E-04
NR_138074	*PAXX*	1.31	2.48	28.25	2.66E-06	3.47E-04
NR_003086	*HSD17B7P2*	3.72	13.14	2.60	6.35E-06	7.07E-04
NR_027709	*IDI2-AS1*	1.99	3.98	15.78	8.92E-06	8.92E-04
NR_109925	*MIR548XHG*	2.77	6.83	5.31	9.83E-06	9.49E-04
NR_135261	*C1orf61*	2.38	5.20	4.89	1.28E-05	1.16E-03
NR_047580	*TKT*	1.10	2.15	60.13	1.87E-05	1.54E-03
NR_037669	*GGCT*	1.23	2.34	21.86	3.29E-05	2.41E-03
NR_037166	*SOD2-OT1*	1.68	3.21	9.00	4.19E-05	2.79E-03
NR_024611	*HINT1*	1.34	2.54	65.80	1.13E-04	5.99E-03
NR_147091	*TSPAN15*	2.72	6.58	10.85	1.76E-04	8.11E-03
NR_048547	*MGST1*	2.35	5.09	14.83	1.94E-04	8.83E-03
NR_131754	*STARD4*	3.40	10.59	4.79	2.65E-04	1.10E-02
NR_120681	*C8orf59*	0.75	1.68	30.91	4.24E-04	1.55E-02
NR_037651	*HSPB2-C11orf52*	2.80	6.96	3.48	5.34E-04	1.83E-02
NR_134536	*QPRT*	2.33	5.01	7.94	5.71E-04	1.93E-02
NR_027679	*RPAIN*	0.91	1.88	23.59	6.85E-04	2.23E-02
NR_037892	*ZNF695*	1.24	2.35	9.78	6.97E-04	2.25E-02
NR_133632	*HSPB11*	1.15	2.22	32.00	7.19E-04	2.29E-02
NR_146205	*PIBF1*	1.19	2.28	25.11	1.03E-03	2.85E-02
NR_045962	*KRT8*	0.74	1.67	1398.83	1.04E-03	2.88E-02
NR_037894	*ZNF670-ZNF695*	1.17	2.25	10.48	1.08E-03	2.97E-02
NR_144418	*MICAL2*	1.62	3.07	4.82	1.13E-03	3.05E-02
NR_134952	*ITGB1BP1*	0.63	1.55	44.02	1.49E-03	3.54E-02
NR_104219	*PTGES3*	0.54	1.46	212.31	1.54E-03	3.62E-02
NR_133653	*RAB25*	1.18	2.27	64.45	1.59E-03	3.66E-02
NR_104285	*CAST*	1.23	2.35	15.35	2.52E-03	4.99E-02

The RefSeq ID (column 1) is shown alongside the relevant gene name: the logarithm fold change (logFC), fold change (FC), the logarithm of the copies-per-million mapped reads (logCPM), the P-value and False Discovery Rate (FDR) (columns 2-7).

Table 2. Long non-coding RNAs significantly higher expressed in the AMA/rba-AMA blastocysts.

RefSeq ID	Corresponding gene name	logFC	FC	CPM	*p* Value	FDR
NR_126449	*LINC01224*	−3.37	10.31	9.38	2.26E-11	3.96E-08
NR_028346	*TRIM53AP*	−6.06	66.81	3.34	2.38E-09	1.54E-06
NR_037923	*DNAAF4-CCPG1*	−2.22	4.66	54.19	3.89E-08	1.88E-05
NR_110695	*LOC101928372*	−3.35	10.22	3.92	7.69E-08	3.29E-05
NR_146772	*LARP1B*	−1.43	2.69	23.26	3.14E-07	8.94E-05
NR_103485	*PLPP1*	−1.28	2.42	34.54	1.64E-06	2.97E-04
NR_148016	*RCBTB1*	−1.52	2.87	30.48	1.82E-06	2.97E-04
NR_117084	*MKRN1*	−1.06	2.08	59.30	2.57E-06	3.47E-04
NR_135826	*USP7*	−1.05	2.07	125.37	5.04E-06	5.70E-04
NR_104317	*FAM234A*	−2.09	4.25	8.22	9.47E-06	9.35E-04
NR_135082	*TMEM51*	−1.83	3.55	10.48	2.06E-05	1.66E-03
NR_136554	*PFKFB3*	−3.05	8.30	9.38	2.66E-05	2.04E-03
NR_131772	*SIMC1*	−1.65	3.14	8.69	4.05E-05	2.74E-03
NR_134887	*GEMIN7-AS1*	−1.09	2.13	17.15	5.89E-05	3.76E-03
NR_024451	*KDM7A-DT*	−2.96	7.80	2.73	5.97E-05	3.80E-03
NR_136655	*SNAP47*	−0.85	1.80	35.26	7.35E-05	4.47E-03
NR_033959	*SMG1P7*	−2.96	7.77	6.19	1.09E-04	5.87E-03
NR_024071	*PLCD1*	−1.72	3.29	11.63	1.24E-04	6.38E-03
NR_038433	*LINC00330*	−2.23	4.70	4.79	1.36E-04	6.76E-03
NR_037646	*TMX2-CTNND1*	−0.63	1.55	49.87	4.86E-04	1.70E-02
NR_135643	*TMBIM1*	−1.39	2.62	36.25	4.99E-04	1.72E-02
NR_152596	*SNHG15*	−1.05	2.06	78.79	6.16E-04	2.05E-02
NR_104108	*SIGMAR1*	−1.38	2.61	8.75	7.41E-04	2.35E-02
NR_037148	*DNAJC25*	−1.20	2.30	12.30	7.82E-04	2.41E-02
NR_028500	*LDHA*	−0.43	1.35	530.06	8.86E-04	2.56E-02
NR_036682	*BCL7B*	−0.91	1.87	22.94	9.95E-04	2.79E-02
NR_046462	*FAM169A*	−1.15	2.21	14.72	1.11E-03	3.02E-02
NR_073554	*CDK5RAP2*	−0.87	1.83	37.01	1.21E-03	3.19E-02
NR_047549	*SLC25A12*	−2.36	5.13	2.73	1.42E-03	3.46E-02
NR_135853	*FAM219B*	−1.27	2.40	8.82	1.44E-03	3.47E-02
NR_046633	*NUCB1-AS1*	−1.22	2.32	8.69	1.52E-03	3.61E-02
NR_028581	*G6PC3*	−1.09	2.13	9.06	1.65E-03	3.73E-02
NR_073169	*ATG9B*	−1.71	3.26	22.32	1.75E-03	3.89E-02
NR_024123	*PBX3*	−1.58	3.00	5.17	1.80E-03	3.96E-02
NR_037573	*DCTN5*	−0.63	1.55	58.49	1.81E-03	3.98E-02
NR_027783	*SAT1*	−1.57	2.98	7.84	1.88E-03	4.05E-02
NR_038198	*PBX4*	−1.70	3.25	3.48	1.93E-03	4.11E-02
NR_003099	*ZNF273*	−1.14	2.21	7.46	2.03E-03	4.27E-02
NR_103546	*SPECC1L-ADORA2A*	−0.97	1.96	17.27	2.10E-03	4.37E-02
NR_046177	*MAPRE2*	−1.82	3.52	6.63	2.21E-03	4.54E-02
NR_024048	*TAZ*	−1.30	2.47	10.13	2.38E-03	4.79E-02
NR_102404	*NBPF8*	−1.24	2.36	16.91	2.42E-03	4.83E-02

The RefSeq ID (column 1) is shown alongside the relevant gene name, the logarithm fold change (logFC), fold change (FC), the logarithm of the copies-per-million mapped reads (logCPM), the P-value and False Discovery Rate (FDR) (columns 2-7).

Taken together, these lncRNAs may play roles in the upregulation of steroidogenesis-related coding transcripts in the YMA/rba-YMA group, potentially promoting steroidogenesis (Zhang R et al. 2020; Otsuka et al. 2021) and regulating genes encoding inflammatory molecules (Chen et al. 2017).

A similar concept can be applied to oncogenic-related lncRNAs that may modify the expression of certain coding transcripts in the YMA/rba-YMA trophectoderm samples, potentially facilitating blastocyst invasion into the endometrium, using mechanisms similar to those functioning in neoplastic invasion. As described previously, these lncRNAs could activate the enhancers of corresponding genes and/or deactivate/inhibit the sncRNAs that negatively regulate the coding transcripts more highly expressed in the YMA/rba-YMA (vs AMA/rba-AMA) groups. It is possible that numerous trophectoderm lncRNAs are packaged into exosomes for secretion into the extracellular space (Russell et al. 2020). Exosome-related transcripts were significantly more highly expressed in the YMA/rba-YMA (vs AMA/rba-AMA) embryos (Ntostis et al. 2021), suggesting that trophectoderm derived YMA/rba-YMA lncRNAs, could enhance the implantation process, potentially by reaching the endometrial cells *via* secreted blastocyst/trophectoderm exosomes as transport vectors (Homer et al. 2017; Figure 3).

The genes corresponding to the lncRNAs shown in Table 1 (and Supplementary Table 1) were subjected to ontological analysis using DAVID and KEGG databases, with 9 out of the 35 genes present in the KEGG pathway for metabolism (*metabolic pathways*; FDR of 1.2E-3). These genes encode for the isomerases IDI1

and IDI2; the transferases GGCT, MGST1 and QPRT; along with the enzymes HSD17B1, MMAB, PTGES3 and TKT. Steroid hormones synthesised by the preimplantation embryo are important for embryonic development and implantation success potentially affecting lipid metabolism. Hence, the more highly expressed enzymes in the YMA/rba-YMA groups may signify/promote a successful implantation process (Dickmann Z and Dey 1974; Dickmann et al. 1975; Zhang S et al. 2013; Ye et al. 2021).

LncRNAs of enzymes reported in the current study, are involved in cholesterol biosynthesis and/or steroidogenesis. Hydroxysteroid dehydrogenases along with the steroidogenic acute regulatory (STAR) and START-domain proteins play important roles in steroidogenesis (Ye et al. 2021), with the lipid-binding STARD4 participating in cholesterol intracellular transport, playing important roles in cellular metabolism. The high expression levels of these lncRNAs in the YMA/rba-YMA group along with the increased corresponding mRNA levels, suggests that the first may act either by enhancing the expression of the latter or inhibit the sncRNAs that regulate their mRNAs, with the same outcome. The current study reported that *MGST1* lncRNA was approximately 5 times more highly expressed in the YMA/rba-YMA than in the AMA/rba-AMA trophectoderm group. MGST1 is a glutathione transferase involved in cellular metabolism, stem cell development and differentiation and when absent is embryonic lethal (Bräutigam et al. 2018), perhaps by affecting blastocyst differentiation and/or stem cell differentiation during embryo development. The *HSD17B1* lncRNA, participating in cholesterol synthesis/steroidogenesis that is important for early embryo development, was expressed approximately 10 times more highly in YMA/rba-YMA blastocysts than AMA/rba-AMA blastocysts. This lncRNA was associated with blastocysts showing higher implantation success rates potentially enhancing the expression of *HSD17B1* (Ntostis et al. 2015; Ntostis et al. 2019; Ntostis et al. 2021).

Significantly more highly expressed lncRNAs in the YMA/rba-YMA group may enhance the expression of genes supporting embryonic implantation with mechanisms similar to carcinogenesis (Bouckenheimer et al. 2016), suggesting a more invasive profile of the young maternal age blastocysts. Steroidogenesis also promotes tumor growth in the tumor microenvironment by inhibiting anti-tumor immunity (Mahata et al. 2020). The antisense lncRNA *RUZC1-AS1*, for example, competes with miR-744, promoting tumorigenesis in cervical cancer by increasing the expression of the antiapoptotic BCL2 (a miR-744 target) and suppressing apoptosis (Guo et al. 2020). MiR-744 can also induce cell death in ovarian cancer (Kleemann et al. 2018). Taken together, by reducing apoptotic stimuli in the trophectoderm cell, the antiapoptotic mechanism suggested here could enhance implantation success in YMA/rba-YMA blastocysts and support the invasion of the embryo into the endometrial tissue. The transcriptional activator lncRNA, C1orf61 (MIR9-1HG), induces cell migration in hepatocellular carcinoma and was significantly more highly expressed in the YMA/rba-YMA group. Alongside the activated STAT3 and/or Akt pathways, MIR9-1HG may promote tumor growth and facilitate metastasis (Yu et al. 2021). Both pathways are already active in human embryonic stem cells, regulating self-renewal and pluripotency (Hirai et al. 2011), hence when MIR9-1HG lncRNA primarily expressed in brain and testis tissues (Wu C et al. 2009) is present at higher expression levels (~5 times higher in the YMA/rba-YMA blastocysts), the trophectoderm cells of the YMA/rba-YMA group may exhibit enhanced invasive characteristics similar to liver cancer cells, facilitating blastocyst implantation success compared with AMA/rba-AMA blastocysts.

As well as being a molecular biomarker in various cancer types, including ovarian and breast cancer, CD24 acts as a pluripotency marker in human and mammalian cells (Kristiansen et al. 2003; Shakiba et al. 2015; Tarhriz et al. 2019). Expression of *CD24* facilitates cancer invasion by increasing the capacity of the cells to metastasize (Mierke et al. 2011). CD24 is elevated in human embryos during blastocyst formation (Yan et al. 2013), indicating a potential mechanism by which YMA/rba-YMA blastocysts can more efficiently invade the endometrium. In this study, the *CD24* lncRNA (NR_117089) was expressed approximately 12.5 times more in the YMA/rba-YMA compared with the AMA/rba-AMA blastocysts, illustrating its likely importance during blastocyst development and consequently the invasion capacity of the blastocyst (Mierke et al. 2011).

TSPAN15 is an essential subunit of the ADAM10 scissor complex, which is also important for early embryo development (Koo et al. 2020). The lncRNA *TSPAN15* (NR_147091) was expressed approximately 6.5 times more in the YMA/rba-YMA than in the AMA/rba-AMA blastocysts. ADAM10 is involved in cell junctions and blastocyst formation and is essential for preimplantation embryo development (Kwon et al. 2016). A knockout mouse model illustrated that ADAM10 is also involved in embryonic cardiovascular

development causing embryonic death after day 10.5 (Zhang C et al. 2010). Perhaps the role TSPAN15 plays in the formation of cell junctions and its higher expression in blastocysts with an increased capacity to implant into the endometrium is indicative of a greater likelihood of successful mammalian early embryo development (Eckert and Fleming 2008; Estill et al. 2019).

Trophectoderm lncRNA characteristics from advanced maternal age blastocysts

Unlike YMA/rba-YMA, the AMA/rba-AMA blastocysts seem to produce more apoptotic and autophagy-related factors, potentially reducing chances of normal embryonic development and/or implantation (Ntostis et al. 2021). These factors could increase the number of degrading (or degenerating) cells of the blastocyst during apposition at or invasion into the endometrium leading to implantation failure (Hardarson et al. 2012; Ramos-Ibeas et al. 2020). These blastocysts, however, may be rescued on certain occasions where the 'faulty' cells are eliminated. In contrast to the YMA/rba-YMA blastocysts, the current study showed that several of the more highly expressed lncRNAs in the AMA/rba-AMA blastocysts were associated with the suppression of tumor growth/progression by means of apoptosis, mitophagy and autophagy. The silencing lncRNA *LINC01224*, for example, is expressed over 10 times more in the AMA/rba-AMA group, illustrating properties associated with suppression of cancer progression. It is known to suppress cancer by downregulating/silencing miR-330 (Gong et al. 2020), a microRNA, that when elevated, promotes the progression of hepatocellular carcinoma (Xiao et al. 2018).

The lncRNAs reported in the current study to be more highly expressed in the AMA/rba-AMA trophectoderm, correspond to/overlap with certain genes, potentially promoting their functionalities and expression levels. The AMA/rba-AMA lncRNAs overlapping with and potentially regulating tumor suppressor genes, include, for example, *PLPP1* (Tang et al. 2014); *RCBTB1* (Mauduit et al. 2019); *USP7* (Jiang et al. 2021); *PLCD1* (Xiang et al. 2010); *SNHG15* (Tong et al. 2019); *DNAJC25* (Liu et al. 2012); *BCL7B* (Uehara et al. 2015); *SLC25A12* (Alkan et al. 2020); *NUCB1* (Hua et al. 2021); *ATG9B* (Wang N et al. 2017); *PBX3* (Morgan and Pandha 2020); *SAT1* (Ou et al. 2016) and *TAZ* (Grieve et al. 2019; Supplementary Table 2). Other AMA/rba-AMA lncRNAs, however, potentially promote cancer progression, creating a mixed microenvironment merging both tumor suppression and tumor progression factors, including *LARP1B*, that promotes ovarian cancer (Hopkins et al. 2016), as well as *MKRN1 (*Ko et al. 2012*); PFKFB3 (*Shi et al. 2017*)*; *SIGMAR1* (Gueguinou et al. 2017); *LDHA* (Ždralević et al. 2018) and *MAPRE2* (Abiatari et al. 2009; Supplementary Table 2).

Several nonsense-mediated decay (NMD) RNAs were highly expressed in the AMA/rba-AMA blastocysts, including *DNAAF4-CCPG1*, *TMX2-CTNND1* and *SPECC1L-ADORA2A*. Other lncRNAs include the pseudogene *TRIM53AP* and the *LOC101928372* locus, which are respectively 67 and 10 times more highly expressed in the AMA/rba-AMA groups, potentially reflecting regulatory roles in these genomic regions or a potential deregulation of these trophectoderm transcripts in women of increased maternal age (Harries et al. 2011; Anisimova et al. 2020).

Previous studies that investigated the trophectoderm or blastocyst transcriptome with respect to maternal age, mainly focused on protein-coding transcripts, without considering the ploidy status (Kawai et al. 2018) or the pregnancy outcome (McCallie et al. 2019). Our analysis illustrates that the metabolically active competent YMA/rba-YMA blastocysts, contain significantly more highly expressed lncRNAs associated with cholesterol/steroid biosynthetic processes, along with factors involved in tumor development/progression, potentially reflecting invasive properties. The majority of AMA/rba-AMA lncRNAs on the other hand, exhibit tumor suppression characteristics. In line with these findings, embryonic genes are re-expressed in different tumors as previously reported (Monk and Holding 2001; Ben-Porath et al. 2008; Bouckenheimer et al. 2016). Considering that there is a significantly higher probability for younger maternal age (YMA/rba-YMA) euploid blastocysts to implant compared with those derived from women of more advanced ages (AMA/rba-AMA), these processes may delineate normal embryonic development and successful implantation, with the RNAs potentially illustrating the cause or the effect of a series of factors that may be altered during preimplantation embryonic development with maternal age. In future, along with the coding RNAs, these lncRNA targets could be used to develop a diagnostic test (Figure 3) that identifies those blastocysts more likely to implant, suggesting a novel approach that could facilitate work aimed at reducing the number of ART cycles required to achieve pregnancy.

Methods

A high throughput analysis was carried out using the RNA sequencing (RNA-Seq) data reported previously

by Ntostis and colleagues (Ntostis et al. 2021). The data were derived from a total of 32 euploid trophectoderm samples, 10 samples from women of young (YMA; below 30 years old), 16 from intermediate (IMA; 30–39 years old) and 6 from more advanced (AMA; 40 years old or above) maternal age that participated in ART treatment. As described previously, trophectoderm cells obtained from day 5 blastocysts were donated following informed consent from couples attending for ART treatment. The ethical approvals were issued by the Greek National Authority of Assisted Reproduction and the National Health System, A' Administration of the Health District of Attica, Greece, General Children Hospital 'Aghia Sofia' (Reference – Protocol Number: 19964/04-09-2014).

Bioinformatics analysis was performed using FastQC (mAndrews 2010) to assess the quality of sequencing reads with Trim Galore (Krueger 2015) used for automatic adapter sequence removal and trimming. All remaining reads were aligned to the human genome (hg38) using the HISAT2 v2.1.0 aligner tool (Pertea et al. 2016). Unmapped/unpaired reads were removed using Samtools (v1.8) and PCR duplicates were tagged by Picard tools v2.1.1 (Picard toolkit 2010). Using an hg38 annotation file of the human transcriptome as a guide (Kent et al. 2002), StringTie v2.1.4. (Pertea et al. 2015) was employed to detect potential novel coding or non-coding transcripts. Two approaches were employed to assign and quantify read mapping, including the prepDE approach (Python script) (Pertea et al. 2016) that extracted read counts from HISAT2/StringTie in a format that could be inserted into edgeR v3.9 (Robinson et al. 2010) and Rsubread's featureCounts function (Liao et al. 2014) that was used to generate count tables. Differential expression analysis was performed using edgeR with a copies-per-million mapped reads (CPM) of at least 5 and FDR <0.5. This CPM was selected as a balanced approach that could reveal the lncRNA biomarkers with higher likelihood to affect/predict implantation success, without leaving out important biomarkers that could be important in implantation success. Only correctly paired, non-duplicated and uniquely mapped reads were considered.

As reported previously (Ntostis et al. 2021), unsupervised hierarchical clustering was performed on the trophectoderm gene expression data to classify the samples into their reproductive biological maternal age (rba) groups relying on corresponding trophectoderm gene expression patterns (Figure 2). Hierarchical clustering analysis was performed at the whole transcriptome level, including both coding and non-coding transcripts (primarily poly-A), based on the logarithm fold-change (logFC) derived from the differential expression analysis. The current analysis focused on differences between the lncRNAs of young and advanced maternal age groups corresponding to a significantly higher implantation success rate when upregulated in the young group and significantly lower implantation success rate when upregulated in the advanced maternal age group.

According to the mode of interaction between the lncRNA and its target(s), the targeted genes were selected on the basis of the location relationship with their corresponding lncRNAs. Each lncRNA was considered to target/regulate its corresponding overlapping protein-coding gene. This approach increased the probability of the lncRNAs acting in a regulatory manner to their overlapping genes. Ontological analysis of the candidate genes which are potentially regulated by the highlighted lncRNAs was performed using DAVID (Huang et al. 2009a, 2009b) and the KEGG database (Kanehisa et al. 2017). These genes were classified based on certain gene ontologies, potentially illustrating the main pathways affected by their corresponding lncRNAs. The data used in this post-hoc analysis are available at the NCBI Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) - accession number GSE133592. The RNA-Seq data analyzed here has been validated previously by RT-qPCR (Ntostis et al. 2021).

Ethical approval

The ethical approvals were issued by the Greek National Authority of Assisted Reproduction and the National Health System, A' Administration of the Health District of Attica, Greece, General Children Hospital 'Aghia Sofia' (Reference – Protocol Number: 19964/04-09-2014). Informed consent from couples was obtained.

Disclosure statement

Author PN has 2 pending patent applications (Application numbers: GB 2107813.4 and PCT/GB2022/051380). This does not alter the author's adherence to the journal's policies on sharing data and materials. All remaining authors declare no conflicting interests.

Authors' contributions

Contributed to the computational analysis of the RNA sequencing data and the interpretation of the results: PN, GS, DI, SAK, DM; manuscript was written collaboratively

by: PN, DM, SAK, GS, HMP, DI, JH, GK, MT; directed the data analysis, writing and editing of the manuscript: PN, DM, SAK, GS. All authors critically reviewed and approved the final version of the manuscript.

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