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The role of *Kifc1*, *Kchn5* and miRNA-302 on in vitro development in 8-cell, morula and blastocyst stage of mouse embryos

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Abstract

Introduction: Embryo development is characterized by lack of cell cycle check-points and overexpression of core circadian oscillators. On previous report we have identified several genes over-and under-detected at human embryo blastomeres. In this study, we investigated the expression profile of *Kcnh5*, *KIFC1* and miRNA-302 genes at three pre-implantation stages of mouse embryo development. **Material and methods**: Total RNA was extracted from mouse embyos at 8-cell, morula and blastocyst stage. The expression profile of *Kcnh5*, *KIFC1* and miRNA-302 was assessed by RT-PCR and the results were normalized with G6pdh expression levels. **Results:** *Kcnh5* showed absence of expression at all stages, indicating novel mechanisms of cell cycle control during blastomeres divisions. *KIFC1* showed positive expression at all stages, with decreasing levels as the embryogenesis progresses. This finding indicates that *KIFC1* may have more important role at early events. miRNA-302 showed increased levels of expression at all stages, with morula having the highest levels. Therefore, miRNA-302 might play an important role at the events that happen during morula stage such as compaction. **Conclusions:** Cell cycle control of blastomeres at early embryogenesis might be based on different mechanisms compared to somatic cells and more research is needed in order to reveal crucial cycling elements.

Key words: Mouse embryo development, *Kchn5, KIFC1*, miRNA-302, cell cycle control, gene expression

Introduction

Genetic markers of the pre-implantation embryo are a challenging field concerning the quality of embryos. The aim is to achieve higher pregnancy rates, reduce multiple pregnancies and obtain healthier babies. To accomplish this purpose, it is crucial to de-

fine the ability of oocytes to complete meiosis and undergo fertilization in order to produce a healthy embryo which has the potential to progress to the blastocyst stage in vitro and implant to produce healthy offspring. The selection of embryos with higher implantation potential is one of the major challenges in ART. Even though nowadays the majority of scientists use morphological criteria for the evaluation of embryos quality, we need strategies to implement the molecular technology in order to investigate the oocytes and embryos quality, regarding gene's expression[1]. Focusing on identifying the expression profile of each stage could be useful to unravel the mystery of these developmental stages[2,3]. Further understanding of the biological role of expressed genes may expand our knowledge for the oocyte maturation, fertilization and early embryogenesis steps. The practical implications of compiling gene expression information on human oocytes and embryos would be enormous since it could potentially help us to understand and solve problems related to quality of embryos and we will use the genetic profile as a non-invasive procedure of embryos evaluation. The aim of several investigators is the expression analysis and the investigation of the molecular profile across all stages of embryos development, as well as the function of known genes.

We have previously reported that the normal human 8-cell embryo is characterised by lack of cell cycle check-points and overexpression of core circadian oscillators and cell cycle drivers[2,3]. More specifically, circadian molecular components such as CLOCK, PERIOD, CRYPTOCHROME and ARNTL (BMAL) as well as Cyclins A, B, E and MYC of the cell cycle machinery are overexpressed. On the contrary, key cell cycle checkpoints Rb and Wee1 are underexpressed[2,3]. We also examined the expression profile of growth factor families (Tgfb, Fgf, Egf) and cytokines in 8-cell embryos, finding a circadian rhythm-independent modulation of such genes in early embryogenesis[4].

Additionally, we identified new molecules that participate in early embryonic divisions such as *Kcnh5* (Eag2), a voltage-gated potassium channel and *KIFC1*, a kinesin family member, which were overexpressed at 8-cell human embryos[3]. *Kcnh5* belongs to the ether-a-go-go (eag) potassium channel family and is associated with cell cycle progression via regulating cell volume dynamics[5]. *Kcnh5* was also shown to bind to alpha and beta-tubulin, indicating an important role in the cytoskeleton formation at early embryogenesis[7] Knockdown of *Kcnh5* resulted in inhibition of proliferation, arrest of cells at G1 phase or even triggered apoptosis depending on cell type, indicating a crucial role in proliferation[3,4]. Another important observation is that *Kcnh5* shows low levels of expression in human oocytes following upregulation after fertilization[7].

KIFC1 is a member of Kinecin-14 family which is characterised by minus-end motility on microtubules[8]. This kinesin is associated with elongation and stabilization of spindle at mitosis and meiosis, biogenesis of acrosome during spermatogenesis and survival of cancer cells with extra centrosomes[9-11]. Additionally, *KIFC1* was found overexpressed in syncytiotrophoblast of normal and pathological human placenta, indicating an active role during implantation[12].

MicroRNAs (miRNAs) are well-established gene-expression regulators which participate in cell cycle and developmental processes. The miRNA-302/367 cluster generally consists of five members, miRNA-302a, miRNA-302b, miRNA-302c, miRNA-302d and miRNA-367 and it is ubiquitously distributed in vertebrates, considered an important regulator of cell cycle and embryonic development. More specifically, the miRNA-302/367 cluster is shown to have crucial role in G1/S transition at mouse embryonic stem cells, targeting cyclins and kinases, in order to promote proliferation[13,14]. Additionally, it participates in

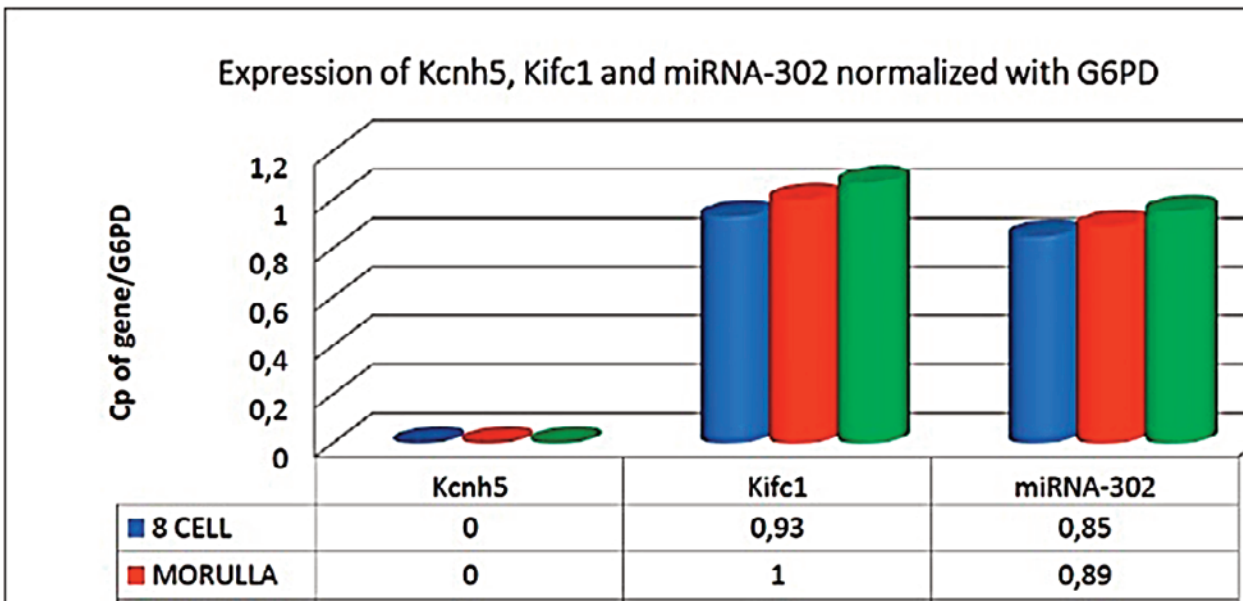

Figure 1. Expression of Kcnh5, Kifc1 and miRNA-302 normalized with G6PD at three developmental stages of mouse embryo, expressed as crossing point (Cp) ratio.

neurulation by suppressing differentiation and neural progenitor expansion[15]. miRNA-302 is also associated with somatic cell reprogramming through global demethylation, indicating a possible role in epigenetic reprogramming of the zygote as well[16]. However, there are conflicting data concerning the expression profile of miRNA-302/367 cluster at embryos. Viswanathan et al found insignificant expression levels of miRNA-302 family during early embryogenesis while Card et al showed that miRNA-302 is expressed at developmental stages E6.5, E7.5 and E8.5[17,18].

On previous report, we have identified 35 cell cycle genes over-detected on whole human genome microarrays of 8-cell stage blastomeres and ten genes under-detected on 8-Cell arrays[2]. Therefore, the objective of this study is to investigate the expression profile of *Kcnh5*, *KIFC1* and miRNA-302 at three pre-implantation stages of mouse embryo development (8-cell, morula and blastocyst). The relative expression of these genes in mouse embryos will reveal which cycling elements are the most crucial for development.

Materials and Methods

Embryo collection and Culture

F1 (C57BL6 x CBA) females 3-4 week-old, were superovulated by 5 IU PMSG (Sigma Chemical Co) followed by 5 IU of hCG (Sigma Chemical Co) 48 hours later. Females were housed individually overnight with males F1 9-week-old and sacrificed 20 hours after hCG administration. Subsequently the oviducts, containing the fertilized oocytes, were removed and cultured at DPBS (Dulbecco's Phosphate Buffered Salin, Gibco) culture medium with 10% BSA (Sigma-Aldrich). The oviducts were incised and the fertilized oocytes were removed, incubated with hyaluronidase, separated from cumulus cells and cultured at 1 ml Ham's (Ham's F10 Medium (-) Hypoxanthine, Gibco)

culture medium with 5mg/ml BSA. The embryos, at groups of forty, were incubated (37 oC, 5% CO2, 95% humidity) for 48 (8-cell stage), 72 (morula stage) and 96 hours (blastocyst stage).

All female and male mice used in this study were C57BL/6 female x CBA male F1 hybrids raised and cared for at the Pasteur Institute (Athens, Greece).

RNA and cDNA preparation

Total RNA was extracted from embryos of 8-cell, morula and blastocyst stage. Total RNA extraction kit was obtained from Qiagen (RNeasy micro kit, Qiagen) and the extraction was performed according to manufacturer's protocol. RNA concentration of each sample was determined by spectrophotometry and its quality was evaluated by agarose gel electrophoresis.

Twenty nanograms of total RNA were used for first-strand cDNA synthesis using random hexamer primers and Superscript II Reverse Transcriptase (Superscript II First Stand Synthesis System for RT-PCR, Invitrogen) according to the manufacturer's protocol. The resulting cDNAs were stored at -20°C.

Real-time PCR

The expression of *Kcnh5*, *KIFC1* and miRNA-302 gene were assessed at three pre-implantation stages of mouse embryo development (8-cell, morula and blastocyst) by real-time RT-PCR using primers and probes pairs particularly synthesized by TIB-MOLBIOL (Berlin, Germany) for this study (Table 1).

The specific primers and probes were used at a concentration of 20 pmol/μl in each reaction. Quantitative real-time PCR was performed in a final reaction volume of 20 μl in a LightCycler 480 white 96-multiwell plate (Roche Diagnostics, Mannhein, Germany). All samples were run in duplicate, and no-template controls were included in all runs to exclude possible DNA contamination. The RT-PCR mixture for *Kcnh5* and *KIFC1* contained 5x LightCycler 480 Genotyping Master (Roche), 0.5 μl for each primer, 0.2 μl for each probe, up to 20 μl total volume of reaction H_2O of LightCycler 480 Genotyping Master (Roche), and 5 μl cDNA. The RT-PCR mixture for miRNA-302 contained 2x QuantiTect SYBR Green Master Mix (Qiagen), 0,4 μl for each primer, up to 20 μl total volume

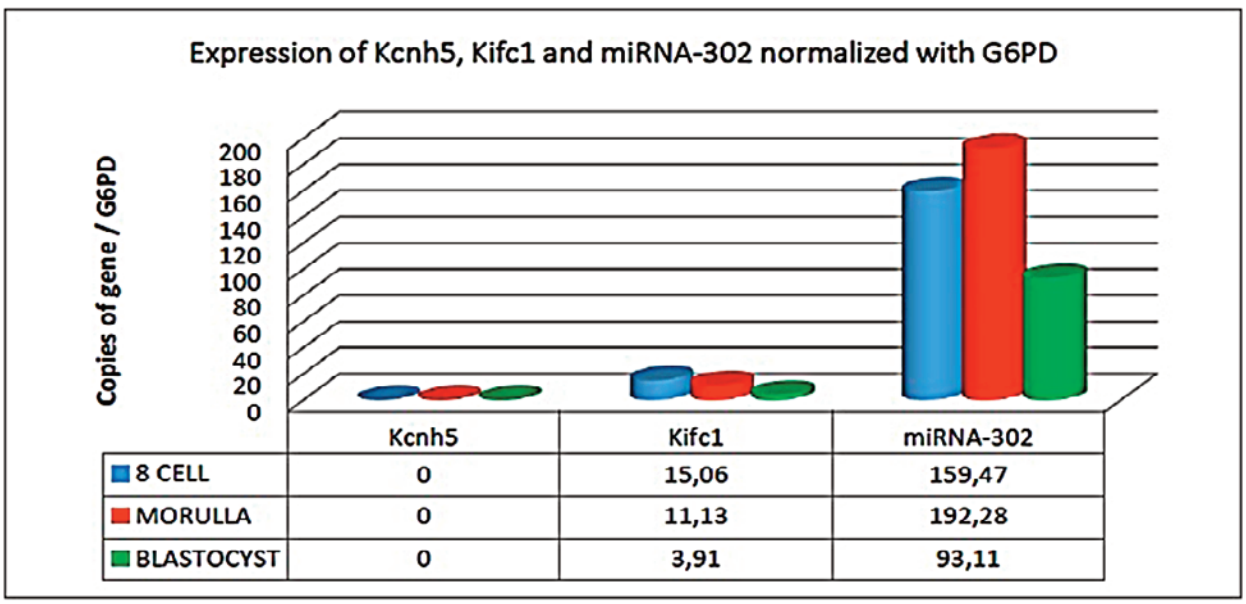

Figure 2. Expression of Kcnh5, Kifc1 and miRNA-302 normalized with G6PD at three developmental stages of mouse embryo, expressed as copies of gene ratio.

Table I. Primers sense and antisense and probes fluorescein and LightCycler of KCNH5, KIFC1, INCENP, miRNA-302 and muG6PD

Gene	Sequence
KCHN5	
KCNH5 S	ATAGGATTTGGAAACATAGC
KCNH5 A	TAACGGTTGGTGTTGGCA
KCNH5 FL	AAAGAAGAGCCGACCATCATCATG-FL
KCNH5 LC	640-CCACTGAGAACATCTTCTCCACGTCTGT p
KIFC1	
KIFC1 S	GGCGGGTGTGGCTGATTG
KIFC1 A	CCTCTTGAGCCTGCTTGCTGAC
KIFC1 FL	ACTTCACCAGGGCTGCCTTCAGTTC-FL
KIFC1 LC	640-ACGTTCCTCTTCACTTCCAACAAAGGTGGC p
miRNA-302	
miRNA-302 A	CTAGCTCGAGTGAGAGTGCTGTCCAAGTAAG
miRNA-302 S	CCGCGGTACCGCTTATGTCTGTAACCGGGTC
muG6PD	
G6PD S	CCAGATCGGGACCTGCTG
G6PD A	AGGCACTCTCTGTAGGCATTCC
G6PD FL	AGTAGAGAGACCGTGCCCCGAG-FL
G6PD LC	640-ACTTCAATGGCATCAAGTACAACACGACC p

of H2O of LightCycler 480 Genotyping Master (Roche), and 2 µl cDNA. G6pdh PCR mixture contained 5µl G6pdh standard (LightMix Kit G6PDH, TIB MOLBIOL), 5x master mix (LightCycler 480 Genotyping Master, Roche), 16x G6PDH (LightMix Kit human G6PDH, TIB MOLBIOL), 25 mM $MgCl_2$ (LightCycler 480 Genotyping Master, Roche) and up to 20 µl total volume of H_2O of LightCycler 480 Genotyping Master (Roche). In all experiments a no template control was used.

For the expression of *Kcnh5*, *KIFC1* and miRNA-302, G6pdh was used as a reference gene. Real-time PCR was performed on Light-Cycler 480 II (Roche) with the following parameters: one cycle of preincubation at 95 oC for 10 min, 40 cycles of amplification for *KIFC1* and *Kcnh5* genes (95 oC for 10 sec, 62°C for *KIFC1* and 56 oC for *Kcnh5* for 20 sec, and 72°C for 10 sec), while for G6pdh and miRNA-302 genes 45 cycles of amplification were used (95 oC for 10 sec, 59°C for mG6PD and 60 oC for miRNA-302 for 20 sec, and 72 oC for 10 sec) and one cycle at 4°C for cooling. The emitted fluorescence was detected at 640 nm.

Results were obtained as Cp-value and number of copies. Cp-value represents the crossing point at which the sample fluorescence overcomes the background fluorescence. *Kcnh5*, *KIFC1* and miRNA-302 results were normalized to G6pdh ones, based on Müller et al[19]. Multiple dilutions of G6pdh gene were used in order to obtain a standard curve and to verify the sensitivity of the experiment.

Results

In order to determine the expression profile of of *Kcnh5*, *KIFC1* and miRNA-302, we divided the collected embryos of every developmental stage at groups of 10 embryos, resulting in 11 speciments for 8-cell stage, 7 for morula stage and 9 for blastocyst stage. The samples underwent RNA extraction and cDNA synthesis and the genes expression in 8-cell, morula and blastocyst stage was studied using quantitative PCR (real-time PCR). The expression results are presented herein as ratios of gene-of-

interest/G6pdh copies (Figures 1 and 2).

Kcnh5

Kcnh5 expression profile was investigated in approximately 270 mouse embryos at 8-cell, morula and blastocyst stages, resulting in absence of expression at these developmental stages.

KIFC1

KIFC1 expression profile was investigated in approximately 270 mouse embryos at 8-cell, morula and blastocyst stages. The expression of this gene was found as positive in all three developmental stages. More specifically, in 8-cell stage the mean detection was at Cp 34.9 (range 31.5-35) with 30.8 copies (range 11.6-74.9), in morula stage at Cp 34.9 (range 34.5-35) with 26.47 copies (range 11.6-45.6) and in blastocyst stage at Cp 35 with 12.9 copies (range 6.74-23). When normalized with G6pdh expression, the Cp ratio was 0.93 (range 0.87-1.07) and the copies ratio 15.06 (range 1.31-64.57) at 8-cell stage. At morula stage, the Cp ratio was found 1.00 (range 0.86-1.16) and the copies ratio 11.13 (3.39-33.53). At blastocyst stage, the Cp ratio was 1.07 (range 0.88-1.26) and copies ratio 3.91 (range 0.98-18.62).

It was found that at 8-cell stage *KIFC1* expression is higher than the subsequent developmental stages with a decrease of gene expression up to 3 to 4 fold at the blastocyst stage.

miRNA-302

miRNA-302 expression profile was investigated in approximately 270 mouse embryos at 8-cell, morula and blastocyst stages. The expression of this gene was found as positive in all three developmental stages. In 8-cell stage, the mean detection was at Cp 31.71 (range 29.18-33.59) with 325 copies (range 66.2-392), in morula stage at Cp 31.07 (range 30.18-31.58) with 385 copies (range 61.5-976) and in blastocyst stage at Cp 31.23 (range 30.74-31.53) with 539 copies (range 61.5-976). When normalized with G6pdh expression, the Cp ratio was 0.85 (range 0.79-0.96) and the copies ratio 159.47 (range 1.15-338) at 8-cell stage. At morula stage, the Cp ratio was found 0.89 (range 0.76-1.04) and the copies ratio 192.28 (10-841). At blastocyst stage, the Cp ratio was 0.95 (range 0.79-1.12) and copies ratio 93.11 (range 16-195).

miRNA-302 expression was detected at all developmental stages and more specifically there was a slight increase of copies from 8-cell to morula stage and subsequently a decrease at blastocyst stage.

Discussion

Embryonic development is a complicated multifactorial process determined by maternally expressed genes switching to embryonic genes transcription. Microarray technology studies helped to clarify which genes are expressed during these developmental processes. It seems that the 8-cells stage is essential for the activation of embryonic genes transcription at human embryos. It is also known that the genes mainly activated are associated with the cell cycle control. Previously, we had identified several genes over- and underdetected at 8-cell stage blastomeres[2].

Cell cycle is a complicated process, under the control of several overlapping mechanisms. It is well established that ion channels contribute to cell cycle control via regulation of the transmembrane potential [20]. More specifically, potassium ion channels regulates the resting membrane potential and it is shown that their function is crucial for cell cycle progression since wide-spectrum potassium channel blockers inhibit proliferation[21].

Kcnh5 gene encodes Kv10.2 protein (eag2) which belongs to the ether-a-go-go (eag) potassium channel family. This ion channel family participates in several important cellular processes such as regulation of resting membrane potential, cellular volume, proliferation

and oncogenesis[5]. *Kcnh5* channel seems to participate at the early events of mitosis via regulation of cell's volume[22]. Bracey et al showed that *Kcnh5* protein binds to alpha and beta-tubulin indicating that this protein might participates in cytoskeleton formation [6]. This finding, in addition to our previous study that showed increased expression at human 8-cell embryonic stage as well as that knockdown of *Kcnh5* resulted in inhibition of proliferation, arrest of cells at G1 phase or even triggered apoptosis, indicates that *Kcnh5* might play a crucial role in proliferation and more specifically in cytoskeleton formation[3,5]. Therefore, we investigated the expression profile of this protein in three developmental stages of mouse embryos. Surprisingly, there was absence of expression at mouse 8-cell, morula and blastocyst stage. This indicates that although *Kcnh5* could contribute in cell cycle control of cell undergoing the usual mitosis process, during blastomere cleavage and blastocyst formation there are other mechanisms of cell cycle control activated independent of potassium channel proteins.

KIFC1 is a member of Kinecin-14 family characterised by minus-end motility on microtubules[8]. It is the only member of this family in mammalian cells[23]. This kinesin is associated with elongation and stabilization of spindle at mitosis and meiosis, biogenesis of acrosome during spermatogenesis and survival of cancer cells with extra centrosomes[9,11]. Additionally, *KIFC1* is crucial for bipolar spindle formation and genomic stability[9]. During embryogenesis *KIFC1* was found overexpressed in syncytiotrophoblast of normal and pathological human placenta, indicating an active role during implantation[12]. In previous study, Kiessling et al showed that this gene is overexpressed at human 8-cell embryos while from our unpublished data it seems that there might be correlation between the levels of *KIFC1* expression and morphology score of human 8-cell embryos[3]. Therefore, we investigated the expression levels of *KIFC1* in three developmental stages of mouse embryos, finding positive expression in all stages. At 8-cell stage, *KIFC1*/G6pdh ratio counted 15.06 copies, being the highest expression level, at morula stage 11.13 copies and at blastocyst stage 3.91 showing a dramatic approximately 4-fold decrease in *KIFC1* expression levels. The increased expression levels of *KIFC1* at mouse 8-cell embryos shows that this kinesin might be an important contributor to the early blastomere cleavage process. But when the proliferation turns to a more cell cycle-controlled procedure such as in blastocyst, the *KIFC1* expression levels decrease and its role probably diminishes.

miRNA-302 is an important regulator of cell cycle since it was found to contribute to cell cycle regulation of embryonic stem cell (ESC) by targeting cyclin D1 and CDK4[14]. Additionally, the miRNA 302/367 cluster was found to have a crucial role in G1-S phase transition by promoting proliferation in mouse ESC[17]. Although, miRNA-302 cluster seems to be an important regulator of cell cycle and is highly expressed in undifferentiated ESCs, there are conflicting data concerning its expression profile during early embryogenesis. Viswanathan et al did not find significant levels of expression at early embryos[17]. On the other hand, Card et al showed that miRNA-302 is expressed at developmental stages E6.5, E7.5 and E8.5. Therefore, we investigated the expression levels of miRNA-302 in three developmental stages, finding positive expression in all of these stages. At 8-cell stage, miRNA-302/G6pdh ratio counted 159.47 copies, at morula stage 192.28 copies, being the highest expression level, and at blastocyst stage 93.11 copies diminished approximately by half. The expression of miRNA-302 during mouse early embryogenesis is at high levels, indicating an important role for the first divisions of blastomeres. Morula is the stage of highest expression levels while there is a decrease by half at blastocyst, indicating that miRNA-302 might play an important role at the events that happen during morula stage. Therefore, it could be useful to identify the events that correlate with highest miRNA-302 expression during morula stage.

Embryo development in vitro is a long and complicated process, initiation and completion of which involve many factors that act either on the oocyte or the early embryo development. For example, *Kcnh5*, a potassium channel believed to be crucial for early events of mitosis, was shown to be absent during early embryogenesis, indicating that blastomeres use different mechanisms for division from somatic cells. The gene expression profiles which are associated with embryo developmental competence, clearly demonstrate that there are differences in the expression in each individual stage of the specific genes that we investigate in this study. *KIFC1* is believed to play fundamental role in the formation of syncytiotrophoblast of normal and abnormal human placenta involved in the process of implantation. *KIFC1* also, has been shown in our study that is present during early embryogenesis with its expression levels decreasing as the embryogenesis progress, indicating a more important role at early events. While miRNA-302 is an important regulator of cell cycle G1/S transition, it was found at highest levels during morula stage indicating that it might participate in other processes except from cell cycle control during early embryogenesis and could play an important role in embryonic control.

We have to take under consideration that these genes are also involved in reprogramming the somatic cells, indicating a possible role in epigenetic reprogramming of the embryos. The challenge now is to correlate the gene function and regulation to specific events in early embryonic development.

Conclusion

In this study, we investigated the expression profile of *Kcnh5*, *KIFC1* and miRNA-302 at mouse embryos during the developmental transition from 8-cell to blastocyst stage. The *Kcnh5* gene was characterized with absence of expression in all three developmental stages while the *KIFC1* and miRNA-302 expression was detected in all stages.

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Ethics Aproval

This study was reviewed and approved by the University and Hospital Ethics Committee and the Animal Care and Use Committee of the Pasteur Institute.

Competing Interests

The authors declare that they have no competing interests

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