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Transcriptome analysis in blastocyst hatching by cDNA microarray*

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BACKGROUND: Hatching is an important process for early embryo development, differentiation and implantation. However, little is known about its regulatory mechanisms. By integrating the technologies of RNA amplification and cDNA microarrays, it has become possible to study the gene expression profile at this critical stage. METHODS: Pre-hatched and hatched ICR mouse embryos (25 blastocysts in each group were used in the triplicate experiments) were collected for RNA extraction, amplification, and microarray analysis (the mouse cDNA microarray, 6144 genes, including expressed sequence tags). RESULTS: According to cDNA microarray data, we have identified 85 genes that were expressed at a higher level in hatched blastocyst than in pre-hatched blastocysts. In this study, 47 hatching-related candidate genes were verified via re-sequencing. Some of these genes have been selected and confirmed by real-time quantitative RT–PCR. These hatching-specific genes were also expressed at a lower level in the delayed growth embryos (morula or blastocyst without hatching at day 6 post hCG). These genes included: cell adhesion and migration molecules [E-cadherin, neuronal cell adhesion molecule (NCAM), lectin, galactose binding, soluble 7 (Lgals7), vanin 3 and biglycan], epigenetic regulators (Dnmt1, and SIN3 yeast homolog A), stress response regulators (heme oxygenase 1) and immunoresponse regulators [interleukin (IL)-2-inducible T-cell kinase, IL-4R, interferon-γ receptor 2, and neurotrophin]. The immunostaining of E-cadherin and NCAM showed strong and specific localization in hatched blastocyst. CONCLUSIONS: This work provides important information for studying the mechanisms of blastocyst hatching and implantation. These hatching-specific genes may have potential as new drug targets for controlling fertility.

Key words: blastocyst/cDNA microarray/gene expression/hatching/implantation

Introduction

Prior to fertilization, the zona pellucida surrounding the mammalian oocyte acts as a species-specific sperm barrier and is involved in sperm binding. After fertilization, the zona plays a role in blocking polyspermic fertilization, it protects the integrity of the preimplantation embryo during early embryonic development, and also helps its oviductal transport. Before implantation, the blastocyst is maintained within the zona pellucida, which prevents ectopic pregnancy (Soupart and Strong, 1975; O'Sullivan *et al.*, 2001). The development of preimplantation embryo to the blastocyst stage, escape of the blastocyst from the zona pellucida (hatching), and differentiation of the uterus to the receptive state are all essential to the process of implantation (Cohen, 1991; Das *et al.*, 2002). Blastocyst hatching and implantation are the results of a well-orchestrated sequence of events of proteinase activation, cellular adhesion with limited invasion, immune regulatory processes, hormones or growth factor secretion, and epigenetic factors, and are controlled in part by some genetic processes and cross-talk between embryo and maternal endometrium (Carlone and Skalnik, 2001; Hill, 2001; Paria *et al.*, 2002). However, the gene regulation in this process remains unclear.

In the past few decades, only a few factors could be studied in each experiment. Since the cDNA microarray technique has been developed in the mid-1990 s, this high-throughput tool has been the most powerful technique in answering the physiological or pathophysiological questions (Chen *et al.*, 1998; Schneider *et al.*, 2004). We have reported the differential gene expression profiles between early gestational decidua and chorionic villi using cDNA microarray technology (Chen *et al.*, 2002). Using this technique, the endometriosis-related genes have also been identified in our previous study (Yang *et al.*, 2004). The global gene profiles of human and mice endometrium during the window of implantation have also been investigated (Reese *et al.*, 2001; Kao *et al.*, 2002). However, a successful implantation not only depends on the maternal endometrium, but also the embryo. To the best of our knowledge, the gene expression pattern in the embryo during the blastocyst hatching stage for implantation remains undiscovered.

Recently, failure of the embryonic zona pellucida to rupture following blastocyst expansion has been put forward as a possible contributing factor in implantation failure. In order to help embryos escape from their zona during blastocyst expansion, different types of assisted hatching have been developed (Cohen, 1991; De Vos and Van Steirteghem, 2000). Early loss of pregnancy after hatching and implantation is also very high, estimated at 25–40% (Wilcox *et al.*, 1988). What is wrong with this process? Although many losses involve genetic abnormalities, there is often no known cause. Several factors, including trypsin-like proteinases, hormonal factors, leukaemia inhibitory factor and prostanoid pathways, might play important parts in successful hatching and implantation (Simpson, 1980; O'Sullivan *et al.*, 2001). But, given the complexities of early development, it is likely that many other genetic and/or epigenetic (DNA methylation) regulatory mechanisms are also involved (Carlone and Skalnik, 2001; Norwitz *et al.*, 2001). In order to study these, the gene expression profile of normal embryo development during the hatching process has become more important.

Preimplantation embryo development, especially the formation of blastocyst, has been studied extensively over the past decade. These studies were focused on specific molecules or a few members of a given family such as nitric oxide (NO)-related factors (Chen *et al.*, 2001; Sengoku *et al.*, 2001), cytokines/hormones (Harvey *et al.*, 1995; Diaz-Cueto and Gerton, 2001; Das *et al.*, 2002), proteinases (O'Sullivan *et al.*, 2001; Whiteside *et al.*, 2001), and signal transductions (Armant *et al.*, 2000) using a one-by-one approach. To overcome the limitation of trace amounts of RNA from a certain number of embryos, the T7 RNA polymerase-based *in vitro* linear RNA amplification was used to amplify the mRNA from blastocyst (Schneider *et al.*, 2004). Herein, the global transcriptomic analyses of blastocyst before or after hatching were investigated by cDNA microarray.

Materials and methods

Embryo collection and culture

This study was approved by the Institutional Review Board, the Animal Care and Use Committee of the Taipei Medical University (Taipei, Taiwan). The ICR mice embryos were collected and cultured in human tubal fluid (HTF medium; Santa Ana, CA, USA) containing 0.3% of bovine serum albumin (BSA, Sigma) as reported previously (Chen *et al.*, 2001). The pre-hatched blastocysts were collected on day 4.0, and the hatched blastocysts were collected on day 4.5 following hCG treatment. The delayed or 'slow' embryo (arrest morula and unhatched blastocyst) were collected until day 6 post hCG. At least 200 embryos were collected and randomly distributed, with ~25 embryos used in each group of the triplicate experiments. These embryos were collected for RNA extraction and amplification (*in vitro* transcription) for cDNA microarray analysis.

In vitro transcription of amplified antisense RNA (aRNA)

Total RNA was extracted from each set of blastocysts using RNAzol™ B reagents (Life Tech, Gaithersburg, MD, USA) and linearly amplified (independently) an estimated 10^6-fold using T7 RNA polymerase as previously and according to the manufacturer's specifications from MessageAmp™ aRNA Kit (Ambion Inc., Austin, TX, USA) (Polacek *et al.*, 2003; Wang *et al.*, 2003; Schneider *et al.*, 2004).

Microarray system

Preparation of cDNA targets and microarray hybridization

Five micrograms of the aRNA derived from blastocysts before or after hatching were labelled with biotin during reverse transcription. All hybridization experiments were performed in triplicate. The details of target preparation, hybridization and colour development have been described previously (Chen *et al.*, 1998; Hong *et al.*, 2000; Chen *et al.*, 2002, 2004a). The 6144 mouse expressed sequence tag (EST) clones with a putative gene name for the mouse cDNA microarray were obtained from the IMAGE consortium libraries through its distributor (Research Genetics, Huntsville, AL, USA). These mouse IMAGE clones were derived from various tissues and in different library constructs, including unfertilized oocytes, whole embryos (from 2-cell to blastocyst), inner cell mass, embryonic stem cells and germ cells, and several other organs (brain, heart, liver, and so on) in different development stages (http://image.llnl.gov/image/html/muslib_info.shtml#NIH_MGC_256). Most of the clones have been partially sequenced and verified, and the sequence information is available as EST from dbEST of GenBank. It has been used in several other mice studies and has been published in our recent works (Chen *et al.*, 2004b; Yu *et al.*, 2004).

Image processing and digitization

After colour development, the microarray images were scanned and digitized using a flat-bed scanner (PowerLook 3000; UMAX, Taipei, Taiwan) (Chen *et al.*, 2004a,b). The scanner provided a high resolution and was suitable for larger arrays such as arrays of 6144 elements. The microarray was processed by commercial image processing programs to convert the true-colour images into gray-scale images, and then the image analysis and spot quantification were done by the GenePix 3.0 (Axon, Union City, CA, USA) or by the MuCDA program, which was written in-house and is available online (http://w3.mc.ntu.edu.tw/department/genechip/supplement.htm).

Real-time quantitative RT–PCR

To confirm the expression patterns of up-regulated or down-regulated genes in the blastocyst hatching process, several re-sequenced and known genes were selected for further analysis using real-time quantitative RT–PCR in a 96-well format as previous described (Chen *et al.*, 2004a). Total RNA from pre-hatched or hatched blastocysts without amplification was used for real-time quantitative RT–PCR. Primers were designed using the Primer Express v2.0 Software (Applied Biosystems Inc., Foster City, CA, USA). All of the primers used in this study have been listed in the Table I. All reactions were carried out in 50 μl volumes containing 25 μl of SYBR Green PCR Master Mix (Applied Biosystems Inc.). The amount of tested gene cDNA relative to the amount of TBP cDNA was measured as $-\Delta CT = -[CT_{Tested\ gene} - CT_{TBP}]$. The ratio of tested gene mRNA copies relative to TBP (TATA box binding protein, used as a housekeeping control) mRNA copies was defined as $2^{-\Delta CT} \times K$ (K: constant).

Immunocytochemistry

The immunostaining protocol was as described in the previous study with some modifications (Chen *et al.*, 2001). Blastocysts were treated in acidic Tyrode solution to lyse the zona, then transferred onto Concanavalin A-coated coverslips, and centrifuged at 180 *g* for 10 min. The embryos were then fixed and incubated with primary antibody (anti-NCAM, anti-E-cadherin, and anti-IL4R; Santa Cruz Biotech Inc., CA, USA) in phosphate-buffered saline (PBS) and 3% BSA. They were then incubated with fluorescein isothiocyanate (FITC)- or rodamine-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotech Inc.) for 60 min at 37°C, counterstained in propidium iodide (PI) or diaminopropidium iodide (DAPI) (Sigma Chemicals) in PBS, and mounted before examination under the fluorescent microscope.

Statistical analysis

Gene expression data obtained from the microarray experiments were processed and normalized using the protocol and programme that has been previously described (Chen *et al.*, 2002). For each gene, the mean and SD of expression as well as the ratios of the mean pre-hatched blastocyst expression versus mean hatched blastocyst expression were calculated and used for comparison. The differentially expressed genes were chosen beyond the 95% predicted regression line. Next, the conventional criteria of 3.0-fold differences were used to sub-classify the significantly different genes. The differentially expressed genes were considered to be significantly down- or up-regulated by a factor of ≥3.0-fold between pre-hatched and hatched blastocysts and were sequence-verified. Expression differences of <2.0- or <3.0-fold have usually been considered at the limit of detection in previous analyses (Popovici *et al.*, 2000; Tanaka *et al.*, 2000; Cavallaro *et al.*, 2002). The genes whose expression corrected with the blastocyst development were grouped into categories by their putative functions on the basis of literature reports. A repeated measure analysis of variance (ANOVA test) was performed to determine any significant difference between the development stages of blastocyst in the real-time quantitative RT–PCR analysis. Where appropriate, the data are expressed as mean ± SEM.

Results

Blastocyst collection and RNA amplification

Almost all embryos achieved the blastocyst stage (containing 64–128 cells) at ~96 h (4 days) post hCG injection. The fully expanded blastocysts started hatching at ~110 h. The blastocysts before and after hatching were collected and divided into two groups (pre-hatched and hatched groups) by

Table I. The primers used for real-time quantitative RT–PCR

Gene name	Primer sequence (forward)	Primer sequence (reverse)
Axotrophin	F-AGGGCAGGGCAGCTGAA	R-TGGCTGAGCGACGACAAC
Endomucin-1	F-TGCAACCACTCCATCAACCA	R-ACAACCAGCGCGATAACCA
HO1	F-TCAGGTGTCCAGAGAAGGCTTT	R-CTCTTCCAGGGCCGTGTAGA
E-cadherin	F-AAGGTGACAGAGCCTCTGGAT	R-ATTCCCGTTGGATGACACA
IL-4R	F-TTAGTGTCAGTGTGGTGCGCTGTA	R-TCTCAGCCTCCAACAAGTCGGAAA
Lactotransferrin	F-GGCCGTCGCGATCTAGAA	R-CAAGAAATATCAAGGAAGGGATGAG
Stanniocalcin	F-CCATCCCCCCTCTCTCTGA	R-TGGTTTGTGTTTGCGGAGAGT
Dnmt1	F-TCAGAGCTGTTCTGTCGTCTGCAA	R-TGAGTCTGCCATTTCTGCTCTCCA
SIN3	F-AGTACAGGGACAGCAGCAGTTTCA	R-AGGGTGGCCTTTAAATAGCTGGGA
Vanin 3	F-TGCAGAGGTTAACTGGAGCGCTTA	R-ACATACACCTCGTCCATTCGGCTT
Dnmt1	F-AAAGTGTGATCCCGAAGATCAAC	R-TGGTACTTCAGGTTAGGGTCGTCTA
Dnmt2	F-TGGTACTTCAGGTTAGGGTCGTCTA	R-TGGTACTTCAGGTTAGGGTCGTCTA
Dnmt3a	F-TGCTACATGTGCGGGCATAA	R-GGAGTCGAGAAGGCCAGTCTT
Dnmt3b	F-CCCAAGTTGTACCCAGCAATTC	R-TGCAATTCCATCAAACAGAGACA
HD-1	F-CTGGGAGGAGGTGGCTACAC	R-GCCACCGCTGTTTCGTAAGT
HD-2	F-GGATGCTGGGAACAGTGCTT	R-AGAGGTTAATGGCAGACTCCTTGT
SIN3a	F-CTCAGATGACCCCGTGGAA	R-CTCGAAGTTCAGGAGAAGTAGTATCAGA
SIN3b	F-TTCAAAAGCCAGAGCATCGA	R-AGGTCAGGGTGCTCATGGAA
PIN	F-TGCTCCACGGTAACCATGTG	R-GTTGCATCTCTTCCGACATGTC
Prtn3	F-CAGCAGAAGTTCACCATCAGTCA	R-GGAGGAGAAGCACGTCATTGA
Pitrm1	F-TGTCCGCCCGCATATTGT	R-GATCTGGGACCCACTGACATGT
Prtn3	F-CAGCAGAAGTTCACCATCAGTCA	R-GGAGGAGAAGCACGTCATTGA
prtse23*	F-TGCTCCACGCGGTAGCA	R-ACAGAGCAGGACAAGAAGGATGA
TBP	F-GCTCACCCACCAACAATTTAGTAGT	R-TGCTTCATAAATTTCTGCTCTGACTT
β-actin	F-CCCTAAGGCCAACCGTGAAA	R-ACGACCAGAGGCATACAGGGA

*prtse23: Mus musculus 12 days embryo female Müllerian duct includes surrounding region cDNA, RIKEN full-length enriched library, clone: 6820428O06 product: SERINE PROTEASE (HYPOTHETICAL 43.0 KDA PROTEIN) (PROTEASE, SERINE, 23) homolog [Homo sapiens].
TBP and β-actin were used as internal control in this study.

the gross morphological examination at the two time-points (Figure 1A). According to this morphological check, the unique groups of the pre-hatched and hatched blastocysts were used for RNA amplification and the following cDNA microarray analysis. Twenty-five embryos per group were used for RNA extraction. The ratio of RNA amplification was ~1000 times, 4.7–13.2 ng of total RNA was extracted from embryos and 5.3–7.9 μg aRNA was obtained after *in vitro* transcription-based RNA amplification. This was similar to previous report (Schneider *et al.*, 2004).

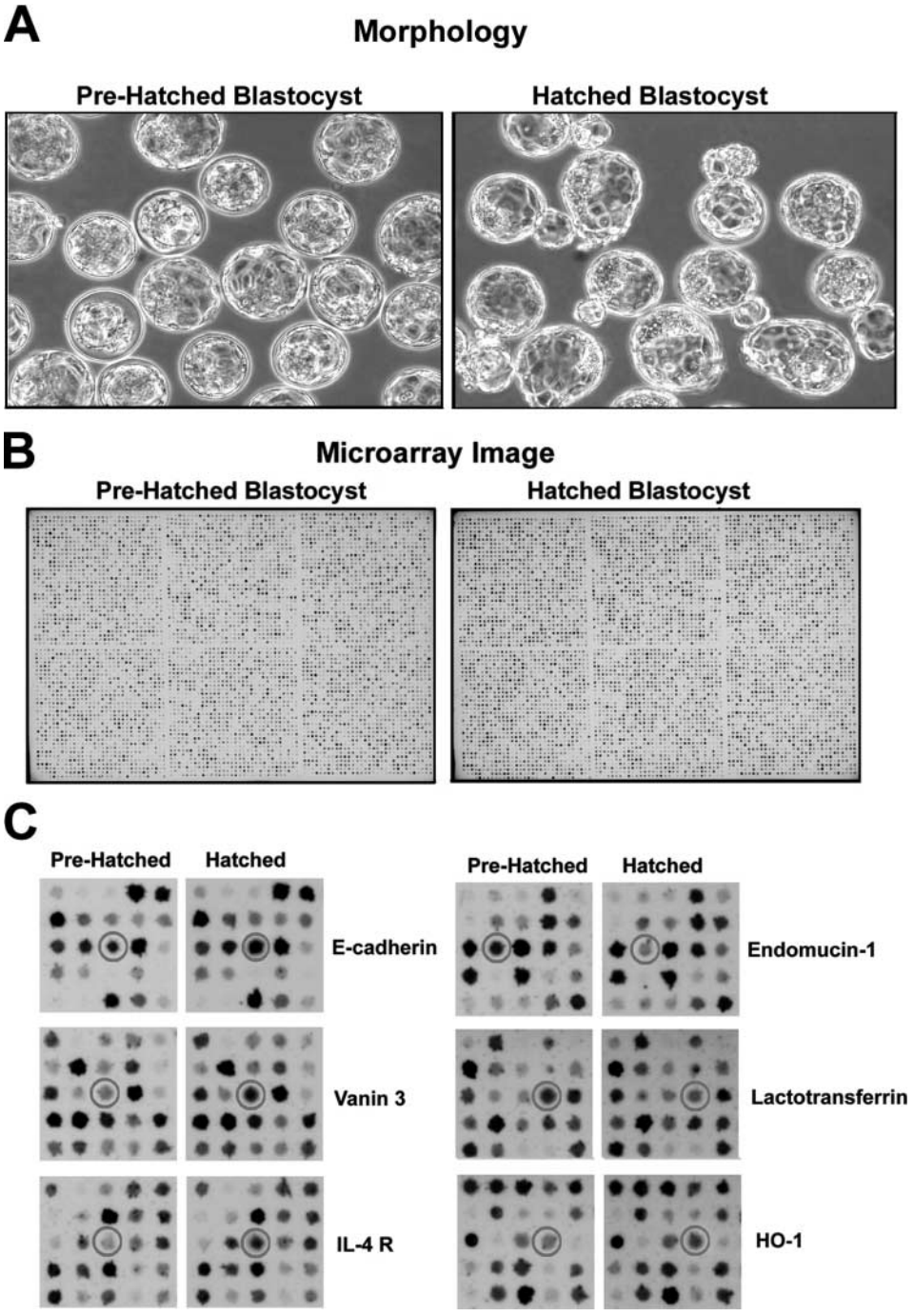

Figure 1. (**A**) Morphological examination of pre-hatching and hatched blastocysts. The pre-hatched and hatched blastocysts were collected for RNA extraction, amplification, and cDNA microarray analysis. (**B**) cDNA microarray analyses of the differentially expressed genes between pre-hatching and hatched blastocysts. Gene expression profiles on cDNA microarray (measured 18 mm × 27 mm) carrying 6144 PCR-amplified cDNA fragments. The digital images of pre-hatched and hatched blastocyst are illustrated. (**C**) Higher magnification views of microarray image showing different gene expression patterns of pre-hatched and hatched blastocysts. The single colour developed dot in the open circles indicates that the mRNA expression levels of E-cadherin, vanin 3, interleukin-4R and heme oxygenase-1 were up-regulated in hatched blastocyst, whereas endomucin-1 and lactotransferrin were significantly down-regulated in hatched blastocyst. Similar results were obtained from three different independent experiments.

cDNA microarray analysis

The gene expression profiles of pre-hatched and hatched blastocysts are shown in Figure 1B. The array signal intensities of pre-hatched blastocyst were compared with those of hatched blastocyst. Figure 1C shows a collection of cropped microarray images (5×5 spots) of the different gene expression patterns between pre-hatched and hatched blastocysts; most of the spots had the same signal intensities between two profiles; however, some of the spots revealed different signal intensities (higher magnification view of the cDNA microarray). In Figure 1C, the cropped microarray images of IL-4R, E-cadherin, vanin 3 and HO-1 were shown in the circles, whose expression levels were higher in hatched blastocyst, whereas the expression levels of lactotransferrin and endomucin-1 genes were higher in pre-hatching blastocyst.

After analysis, it was determined that the expression of 85 detected genes was up-regulated by a factor of ≥3-fold in blastocysts at the hatching stage and only 13 genes were ≥2-fold in blastocysts before hatching. Table II lists the categories of 47 up- and down-regulated known and novel genes which have been verified via re-sequencing, which were significantly altered in hatched blastocysts (Table II). The ratios showed the difference between two stages in mRNA gene expression level. The complete gene expression profiles in both pre-hatching blastocyst and hatched embryo and other supplement data are posted on our website: http://w3.mc.ntu.edu.tw/department/genechip/supplement.htm.

Table II. Some of the up (↑) or down (↓)-regulated genes, which were verified by re-sequencing, in the hatching stage blastocysts

Acc. No.	Title	H:B[a]	Category
XM_156186	Neuronal protein NP25	4.06 ↑	Apoptosis
NM_009864	E-cadherin	3.67 ↑	Cell adhesion/migration
Y00051	Neural cell adhesion molecule	4.10 ↑	Cell adhesion/migration
NM_011979	Vanin 3	4.05 ↑	Cell adhesion/migration
NM_007669	Cyclin-dependent kinase inhibitor 1A (P21)	3.95 ↑	Cell cycle regulator
U83902	Mitotic checkpoint component Mad2	3.83 ↑	Cell cycle regulator
NM_080644	Calcium channel, voltage-dependent, gamma	4.44 ↑	Channel
NM_013456	Actinin alpha	3.84 ↑	Cytoskeleton
AF175432	DNA (cytosine-5)-methyltransferase (Dnmt1)	4.89 ↑	Differentiation
NM_010050	Type 2 deiodinase (Dio2)	3.67 ↑	Differentiation
XM_111892	Butyrate response factor	3.94 ↑	Differentiation/growth factor
NM_008496	Lectin, galactose binding, soluble 7 (Lgals7)	4.08 ↑	Differentiation/growth factor
BC021425	Stanniocalcin	5.45 ↑	Hormone
NM_010215	Interleukin 4 induced 1 (Il4i1)	3.67 ↑	Immunoresponse regulator
NM_010583	IL2-inducible T-cell kinase	4.08 ↑	Immunoresponse regulator
NM_010511	Interferon gamma receptor 2	3.43 ↑	Immunoresponse regulator
BC012309	Interleukin 4 receptor, alpha	3.46 ↑	Immunoresponse regulator
NM_008372	Interleukin 7 receptor	3.53 ↑	Immunoresponse regulator
AF176913	Neurotrophin	4.61 ↑	Immunoresponse regulator
BC052857	Biglycan	3.75 ↑	Matrix proteins
NM_011178	Proteinase 3 (Prtn3)	3.32 ↑	Proteinase
BC006917	Pitrilysin metalloprotease 1 (Pitrm1)	4.13 ↑	Proteinase
BC012708	Proteasome (prosome, macropain) 26S	3.71 ↑	Proteinase
AK078518.1	prtse23	4.52 ↑	Proteinase
BC001491	Heme oxygenase (decycling) 1 (HO1)	3.43 ↑	Stress response
AF020185	Protein inhibitor of nitric oxide synthase (PIN)	3.78 ↑	Stress response
BC058258	Mitogen activated protein kinase 1 (MAPK1)	4.11 ↑	Stress response
AB024004	KRAB-containing zinc-finger protein KRAZ1	4.24 ↑	Transcription factor
U75530	PHAS-II	3.56 ↑	Transcription factor
NM_011378	Transcriptional regulator, SIN3 yeast A	4.24 ↑	Transcription factor
BC039286	Mus musculus RIKEN cDNA A430096B05	3.91 ↑	EST
BC021320	Mus musculus, clone IMAGE:5035544	3.93 ↑	EST
AC124190	Mus musculus BAC clone RP23-267M9	3.08 ↑	EST
AI448386	Mus musculus cDNA clone IMAGE:560499	4.14 ↑	EST
AC118686	Mus musculus chromosome 6, RP23-402P24	3.21 ↑	EST
AK011403	Mus musculus 10 days embryo whole cDNA, clone:2610015J01	4.55 ↑	EST
NM_016885	Endomucin-1	0.46 ↓	Anti-adhesion
BF451445	Apoptosis related protein APR-5	0.47 ↓	Apoptosis
NM_008197	Histone H1	0.34 ↓	Cell cycle regulator
AF045573	FLI-LRR associated protein-1	0.45 ↓	Cytoskeleton/cell migration
NM_020575	Axotrophin	0.41 ↓	Degeneration
NM_010185	Immunoglobulin gamma Fc receptor I	0.37 ↓	Immuno-response regulator
BC006904	Lactotransferrin	0.22 ↓	Iron transport/antibacteria
NM_133365	Axonemal dynein heavy chain 5	0.44 ↓	Microtubules
CF104600	Mus musculus cDNA clone IMAGE:6941421	0.36 ↓	EST
BC079550	Mus musculus cDNA clone IMAGE:6852953	0.45 ↓	EST
BB556122	Mus musculus cDNA clone E330022K08	0.45 ↓	EST

[a]H:B = the ratio of gene expression level of hatched blastocyst to pre-hatching blastocyst.
EST = expressed sequence tag.

Real-time quantitative RT–PCR

To demonstrate that the mRNA expression of identified genes was consistent with the microarray analysis and to avoid the error of aRNA amplification, real-time quantitative RT–PCR with specific primers was used to examine the differentially expressed genes between pre-hatching and hatched embryos with non-amplified RNA samples. Figure 2A shows that 10 selected genes for real-time quantitative RT–PCR analysis have the same trend when compared with the microarray analysis (Figure 2A). The genes heme oxygenase (decycling) 1 (HO-1), E-cadherin (E-Cad), interleukin-4 receptor (IL-4R), DNA (cytosine-5)-methyltransferase (Dnmt1), stanniocalcin (Sta), transcriptional regulator SIN3 yeast homolog A (SIN3), and vanin 3 were all highly expressed in hatched blastocyst; lactotransferrin (LTF), endomucin-1 (Endo-1) and axotrophin (Axot) were expressed at lower levels in hatched blastocyst. These hatching-related genes were also less expressed in the delayed growth

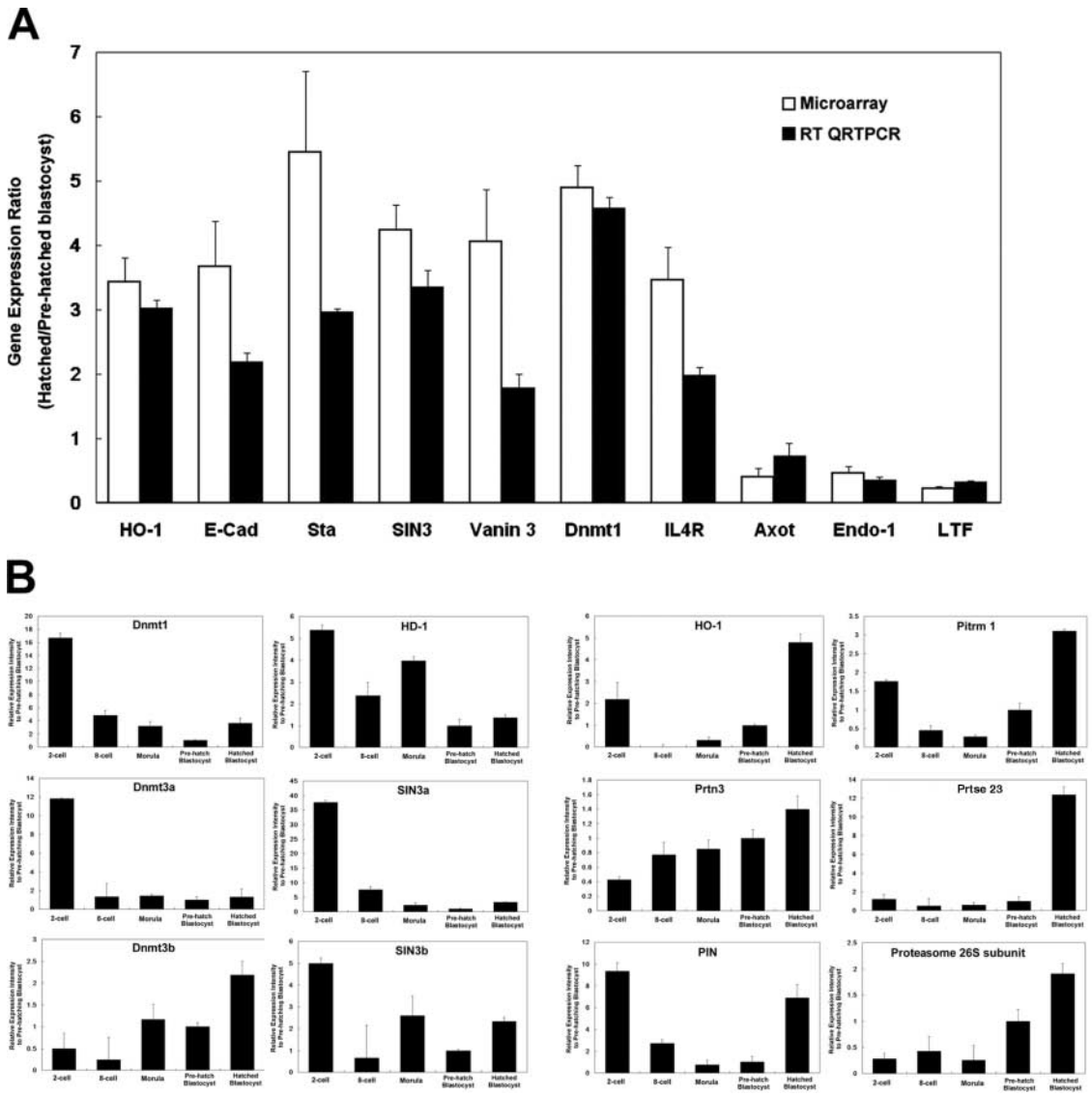

Figure 2. (**A**) Comparison of the data for microarray analysis with real-time quantitative RT–PCR, which confirmed seven up-regulated genes and three down-regulated genes during blastocyst hatching. The fold change is displayed as relative to pre-hatching blastocyst ($n = 4$) and compared with the data from microarray image analysis ($n = 3$) for heme oxygenase (decycling) 1 (HO-1), E-cadherin (E-cad), stanniocalcin (Sta), transcriptional regulator SIN3 yeast homolog A (SIN3), vanin 3, DNA (cytosine-5)-methyltransferase (Dnmt1), interleukin-4 receptor (IL-4R), axotrophin (Axot), endomucin-1 (Endo-1), and lactotransferrin (LTF). (**B**) The mRNA expression profiles of the hatching-related genes were identified at different stages of 2-cells, 8-cells, morula, pre-haching blatocysts, and hatched blastocyst via real-time quantitative RT–PCR. The data were expressed as relative expression intensity to pre-hatching blastocyst ($n = 4$). DNA (cytosine-5)-methyltransferase (Dnmt1); DNA (cytosine-5)- methyltransferase 3a (Dnmt3a); DNA (cytosine-5)-methyltransferase 3b (Dnmt3b); histone deacetylases 1 (HD-1); Mus musculus transcriptional regulator, SIN3A (yeast) (SIN3a); Mus musculus transcriptional regulator, SIN3B (yeast) (SIN3b); heme oxygenase (decycling) 1 (HO-1); proteinase 3 (Prtn3); protein inhibitor of nitric oxide synthase (PIN); pitrilysin metalloprotease 1 (pitrm1); prtse23: *Mus musculus* 12 day embryo female Müllerian duct includes surrounding region cDNA, (PROTEASE, SERINE, 23) homolog [Homo sapiens] (prtse23).

embryos (morula or unhatched blastocyst harvested at day 6 post hCG) (data not shown).

The importance of DNA methylation in early embryo development and several DNA methylation-related genes has been identified in the microarray study. Herein, the gene expression patterns of these DNA methylation-related genes (Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, HD-1, SIN3a and SIN3b) were examined from 2-cell stage, 8-cell stage, morula, pre-hatching blastocyst to hatched blastocyst via real-time quantitative RT–PCR (Figure 2B). The Dnmt1 was extensively expressed at 2-cell stage and declined to very low level at pre-hatching blastocyst, then significantly increased after hatching (3.66 ± 0.72-fold). The Dnmt2 could not be detected during these stages. The Dnmt3a and 3b showed very different expression profiles. Whereas Dnmt3a was sharply down-regulated at the 2-cell stage, Dnmt3b showed an ascending trend from morula to hatched blastocyst (Figure 2B). The histone acetylating-related factors (HD-1 and SIN3b) have higher expression levels at 2-cell stage, morula and hatched blastocyst, as compared with 8-cells and pre-hatched blastocyst. The expression of SIN3a was significantly decreased following 2-cell stage, then increased after hatching (3.37 ± 0.91-fold compared with pre-hatching blastocyst).

The gene expression profiles of three hatching-related proteinases [pitrilysin metalloprotease 1 (Pitrm1), proteinase 3 (Prtn3), and RIKENCDNA6820428-006, similar to human serine proteinase 23 (prtse23)] and other novel hatching-stage expressed factors [HO-1, protein inhibitor of nitric oxide synthase (PIN), and proteasome 26S subunit], which have higher expression level in hatched blastocyst, have also been identified from 2-cell stage, 8-cell stage, morula, pre-hatching blastocyst to hatched blastocyst via real-time quantitative RT–PCR (Figure 2B). The prtn3 and pitrm1 are two novel proteinases, which were up-regulated during the embryo

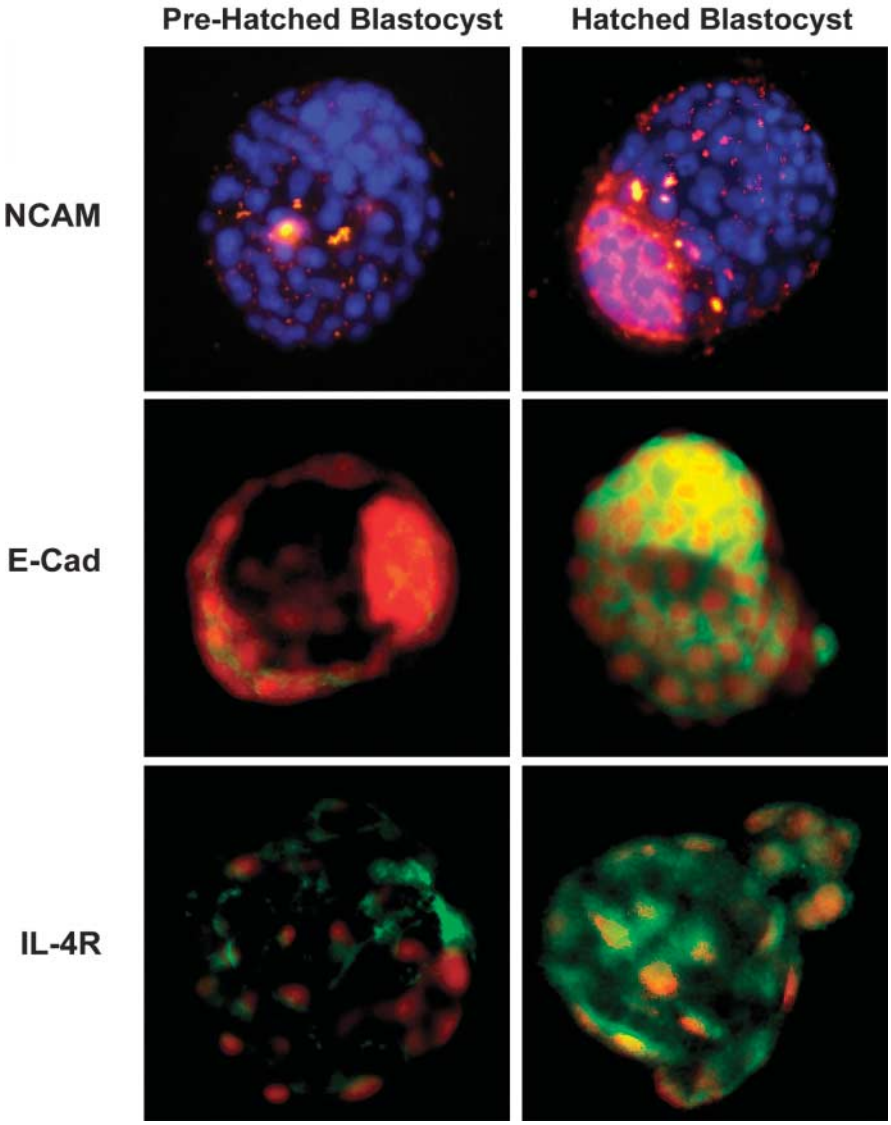

Figure 3. Protein expression level and localization have been identified by using specific antibodies against neuronal cell adhesion molecule (NCAM) (**a** and **b**), E-cadherin (**c** and **d**), and interleukin-4 receptor (IL-4R) (**e** and **f**) followed by the secondary antibody conjugated with rodamine (red fluorescence for NCAM) or fluorescein isothiocyanate (green fluorescence for E-cadherin and IL-4R) in pre-hatching (**a**, **c** and **e**) and hatched blastocyst (**b**, **d** and **f**). Diaminopropidium iodide (blue, in **a**, **b**) and propidium iodide (red, in **c**–**f**) were used as counterstaining for nuclei localization. Magnification ×400.

development and attained a higher expression level at hatched blastocyst as compared with other stages of preimplantation embryo. The novel serine proteinase, 12 days mice embryo female Müllerian duct includes surrounding region cDNA, similar to human serine proteinase 23 (prtse23), was almost undetectable from 2-cell stage to pre-hatching blastocyst, but showed >10-fold increase at hatched blastocyst stage (12.45 ± 0.81-fold). Other hatching-related factors (HO-1, PIN and proteasome 26S subunit) also show the related higher expression level at hatched blastocyst (Figure 2B).

Immunohistochemistry

Figure 3 showed that the protein level and location of NCAM, E-cadherin and IL-4R were highly expressed in hatched blastocyst, and of these, the NCAM was more highly expressed in the inner cell mass side of the hatched blastocyst, which might be the implantation site to the decidual endometrium. The E-cadherin was distributed almost in all blastocyst membrane, but it showed stronger staining in inner cell mass. IL-4R has been found to be expressed in both the pre-hatching and hatched embryo, but the hatched embryo also showed more IL-4R staining.

Discussion

In this study, the differential expression profile of numerous gene transcripts in mouse blastocysts before and after hatching has been identified in large scale by cDNA microarray. The method we describe here, to amplify linearly aRNA from pre-hatched and hatched blastocysts, generated sufficient amounts of materials for microarray analysis. In this study, only 75% of the transcriptomic difference could be confirmed by real-time quantitative RT–PCR, and by the limitation of mouse cDNA library, only 6144 clones had been used in this array (Chen *et al.*, 2004b; Yu *et al.*, 2004). These 6144 mouse IMAGE clones were derived from various tissues and in different library constructs, including unfertilized oocyte, whole embryos (from 2-cell to blastocyst), inner cell mass, embryonic stem cells and germ cells, and several other organs (brain, heart, liver, and so on) in different development stages. However, it is still possible that some of the stage-specific clones during developmental progression might have been omitted in this study. Using this technique, 47 hatching-related genes (including known and novel genes) were identified for their expression patterns during embryo development and hatching, including epigenetic regulators (SIN3 and Dnmt1), cell adhesion or anti-adhesion molecules (E-cadherin, NCAM, vanin 3 and endomucin-1), immunoresponse factors (IL-4R and IL-7R), and some proteinases (pitrm1 and prtn3). Some of these genes have been reported to be critical for oogenesis, preimplantation development, embryo implantation and differentiation, including E-cadherin, NCAM and Dnmt1 (Klementiev *et al.*, 2002; Ratnam *et al.*, 2002), but others have not been reported.

Previously, using suppression subtractive hybridization (SSH) and heterologus cDNA array, the gene expression profiles in mice and bovine oocyte and/or preimplantation embryos have been screened (Dalbies-Tran and Mermillod, 2003; Zeng and Schultz, 2003). Using human cDNA array, ~300 genes have also been identified in bovine oocyte, and of this amount, 70 transcripts were expressed differently during *in vitro* maturation (IVM) (Dalbies-Tran and Mermillod, 2003). The mRNA expression patterns of 10 genes [including Na/K-ATPase alpha1, E-cadherin, zonula occludens protein-1 (ZO-1), glucose transporter-1 (Glut-1), Glut-2, Glut3, and others] have also been studied during blastocyst expansion by RT–PCR (Wrenzycki *et al.*, 2003).

Recently, the global gene expression profiles of mice and human pre-implantation embryos from germinal vesicle (GV) oocyte to blastocyst have already been published via *in vitro* transcription and microarray techniques (Dobson *et al.*, 2004; Hamatani *et al.*, 2004a; Wang *et al.*, 2004; Zeng *et al.*, 2004). The differential expressions of genes between inner cell mass and trophoblast in blastocyst were also reported (Dreesen *et al.*, 2002). For the first time, the blastocyst hatching-specific gene expression profile was identified in this study. Here, we compared the previous reports and ours to focus on discussing the hatching-related genes, including cell adhesion molecules, epigenetic regulators, immunoresponse modulators and hatching-related proteinases, which were and/or might be related to some critical events during embryo development, hatching, cell differentiation and implantation.

Cell adhesion molecules (CAM) are important during embryo development not only in cell–cell adhesion to maintain the structure of blastocyst, but also in cell–cell interaction and communication in embryo implantation (Aplin, 1997; Kimber and Spamswick, 2000). There were at least four adhesion molecules (NCAM, E-cadherin, galatin 7 and vanin 3) up-regulated after blastocyst hatching in this study, and the immunohistochemical staining also showed that NCAM and E-cadherin were localized in the inner cell mass of the embryo, which might also be the site for implantation (Duc-Goiran *et al.*, 1999). NCAM not only plays an important role in neural migration, differentiation and nervous system development, but also as a survival factor against teratogen pyrimethamine (Klementiev *et al.*, 2002). E-Cadherin deletion might lead to development defects in several development stages and has been suggested as playing the critical role in embryo implantation (Larue *et al.*, 1994). Vanin 3 and galatins (1, 3 and 4) have been reported to be involved in preimplantation embryo development and blastocyst activation (Hamatani *et al.*, 2004a,b). Galatin3 has been reported to be expressed at higher levels in blastocyst compared with morula (Ponsuksili *et al.*, 2002). Herein, according to the microarray data, the vanin 3 and galatin7 were both up-regulated at hatched blastocyst, which might suggest that these two adhesion molecules could play a role in blastocyst adhesiveness for embryo implantation. Further studies are underway to identify the detailed mechanisms of these CAM in regulating cell–cell connection within the embryo or between embryo and maternal endometrium.

Epigenetic modification of the genome could regulate several critical biological and pathological events, including development and carcinogensis (Li, 2002; Jones, 2002). Epigenetic reprogramming was thought to be an important issue during mammalian development (Reik *et al.*, 2001).

There are two major events to regulate this epigenetic modification, DNA methylation and histone acetylation. Several epigenetic regulator-related events (including DNA methyltransferase and histone deacetylase) have been identified in early embryo development. The DNA methyltransferase family (Dnmt1o, Dnmt1, Dnmt2, Dnmt3a, and 3b) has been shown to be involved in oogenesis, embryo development, and cell differentiation (Ding and Chaillet, 2002; Ratnam *et al.*, 2002). Previous studies have shown that the DNA methylation was decreased after fertilization, increased after blastocyst, and maintained during fetal development (Reik *et al.*, 2001). Dnmt1o was thought to be critical for oogenesis and Dnmt3a and 3b for embryo differentiation (Bird, 1999; Reik *et al.*, 2001). In the hatching process, the blastocyst transforms from low-methylated to higher-methylated status. In this study, Dnmt1, Dnmt3a and Dnmt3b were found to be up-regulated after blastocyst hatching, which might suggest that these Dnmt could be the epigenetic reprogramming regulator during this reverting process. The results are consistent with previous reports of the epigenetic regulation during the preimplantation embryo development (Bird, 1999; Reik *et al.*, 2001). Other epigenetic regulators, SIN3a and SIN3b, were also increased after blastocyst hatching. The SIN3 complex shares four core proteins with NuRD (HD-1, 2, RbAP46 and 48) and SIN3 is proposed to act as a scaffold for the complex and this complex might be modulated by nuclear hormone receptor, which might play the role in histone deacetylation and lead to DNA methylation (Ahringer, 2000). Recently, a role for SIN3 was demonstrated in cell survival, cell cycle regulation, as well as a regulatory role in mitochondrial respiration (Pile *et al.*, 2003). These results seem to indicate that Dnmt1, Dnmt3a, Dnmt3b, SIN3a and SIN3b might be the important epigenetic regulators in the blastocyst after the hatching process and might direct subsequent embryo development, implantation and differentiation.

Nitric oxide (NO) and carbon monoxide (CO) are novel gaseous chemical messengers that play key roles in cell function and cell–cell communication in many organ systems, including the reproductive system. Although the presence of NO synthase (NOS) in development and its role in the regulation of embryo growth and apoptosis are well established (Shaul, 1995; Chen *et al.*, 2001), little is known about the expression and activity of heme oxygenase (HO), the enzyme that catalyses the oxidation of heme to CO, biliverdin and iron, during preimplantation embryo development. In this study, HO-1 has been found to be up-regulated during blastocyst hatching. This might suggest that the emzyme or its metabolites could be the regulator for embryo hatching or a survival factor for the hatched embryo. HO-1 has been reported to prevent CD95/FasL-mediated apoptosis, as an immunoregulator, which could significantly prolong allogeneic orthotopic liver transplantation survival via a downstream HO-1–CO signalling pathway (Ke *et al.*, 2002).

Before implantation, the blastocyst is maintained within a proteinaceous coat, the zona pellucida, which prevents polyspermy and ectopic pregnancy. An extracellular trypsin-like activity, the proteinase enzyme, is necessary for the hatching process. Previously, a novel murine tryptase, implantation serine proteinase (ISP1) gene, has been reported (O'Sullivan *et al.*, 2001). In this study, three novel proteinases were found during the blastocyst hatching process. The microarray data and real-time quantitative RT–PCR show that the Pitrm1, Prtn3 and Prtse23 genes were expressed at the blastocyst stage and dominant at the hatched blastocyst stage. These early expressed proteinases might play a role in the embryo hatching and implantation processes.

In summary, blastocyst hatching is an important developmental process for embryo implantation, whereas assisted hatching in many cases is indicated in assisted reproduction and probably enhances clinical pregnancy in older women (Cohen, 1991; Edi-Osagie *et al.*, 2003). This study provides the last piece of the map to complete the profiles of the dynamic gene expression changes from GV oocyte to hatched blastocyst by using the T7 RNA polymerase-based *in vitro* linear RNA amplification and cDNA microarray, as compared with previous reports (Dobson *et al.*, 2004; Hamatani *et al.*, 2004a; Wang *et al.*, 2004; Zeng *et al.*, 2004). Our study has pointed out the usefulness of investigating the regulatory mechanisms and has selected some candidate genes in blastocyst hatching and further implantation. Not only cell adhesion molecules, but also epigenetic regulators, immunoresponse modulators, survival factors, and hatching-related proteinases might play important roles in this critical process of embryo development. This work also provides information for studying these hatching-specific genes which may become new drug targets for controlling fertility.

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