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# LAT1 Expression in Pre and Post Implantation Embryos and Placenta

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## Abstract

**Objectives—**LAT-1 (L-type amino acid transporter 1) is a system L, Na+-independent amino acid transporter responsible for transport of large neutral amino acids. Dysregulated expression of LAT-1 is characteristic of many primary human cancers and is related to tumor invasion. Primary rat hepatocytes in culture increase LAT-1 mRNA in response to amino acid depletion. Transformed hepatic cell lines demonstrate constitutive expression of LAT-1. These observations suggest that LAT-1 expression confers a growth and survival advantage under limited amino acid availability. LAT-1 is highly expressed in the placenta. It has been shown previously that amino acids are fundamental regulators of cell function and energy metabolism in pre-implantation embryos. Our objectives were to analyze qualitatively and quantitatively LAT-1 expression in pre-implantation stages of mouse embryo development and to identify cell types expressing LAT-1 in post implantation stages.

**Methods—**LAT-1 was quantified by real-time qPCR. Localization of expression was by laser capture microdissection, *in situ* hybridization and immunohistochemistry.

**Results—**Our results show increasing mRNA levels of LAT-1 as the embryo develops from zygote to blastocyst with highest levels at hatching blastocyst. Expression studies of LAT-1 on microdissected samples from developing mouse placenta show highest levels of LAT-1 mRNA in trophoblast giant cells (TGC's) at the time of implantation (E7.5), followed by maternal decidua, ectoplacental cone and epiblast. At later stages of development (E9.5 and E11.5) no differential expression of LAT-1 was observed. In *situ* hybridization and immunohistochemistry also showed differential expression of LAT-1 mRNA and protein, respectively, with darkest staining in TGC's at E7.5. By E9.5 and E11.5 mRNA expression was no longer preferentially localized to TGC's, hybridization was equal across the different cell types and regions. LAT-1 protein expression, however, still showed highest intensity of staining in TGC's at E9.5 and E11.5.

**Conclusions—**Since trophoblast giant cells are invasive cells that displace and phagocytose the uterine epithelial cells, these data suggest that LAT-1 may play a role in the invasive phenotype. The mechanism of LAT-1 regulation during placentation, therefore, might provide valuable clues to its role in tumor progression and invasion.

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## INTRODUCTION

Amino acid transport across plasma membranes is mediated by transporter proteins. Amino acid transporter systems are characterized according to functional criteria such as substrate specificity and sodium dependence [1]. Over a dozen of these transporter systems have been identified in the placenta [1,2]. LAT-1 (L-type amino acid transporter 1) is a $Na^+$-independent amino acid transporter and belongs to the amino acid transport system L. System L is a major nutrient transport system responsible for the transport of large neutral amino acids including several essential amino acids [3–5]. For plasma membrane transport, LAT-1 must heterodimerize with the heavy chain of the cell surface antigen 4F2 [5,6]. 4F2 was originally identified as an activation antigen of lymphocytes and forms a heterodimer consisting of the type II membrane glycosylated protein of around 80 kDa heavy chain, 4F2hc, and a non-glycosylated lighter chain proteins of around 40 kDa linked by a disulfide-bond [7,8]. It has been shown that LAT-1 corresponds to a 4F2 light chain (4F2lc) [5,9–10].

LAT-1 is identical to TA1, tumor associated gene 1 [10]. TA1 was identified by expression screening with a monoclonal antibody [11]. Whereas 4F2hc is widely expressed [5], LAT-1 expression is highly tissue specific. It is expressed in restricted sites such as brain, fetal liver, bone marrow, spleen, testis, ovary and placenta [3,5–6]. Investigations have shown that LAT-1 may play an important role in carcinogenesis. Over expression of LAT-1 is characteristic of many primary human cancers and may be related to tumor progression [12–14]. Studies of rat primary hepatocyte cultures demonstrated that LAT-1 RNA levels were increased in response to amino acid depletion. Upregulation of LAT-1 following amino acid restriction is associated with enhanced protein synthesis, shortened cell cycle progression and enhanced proliferation, suggesting adaptive nutrient regulation [15–16]. In contrast, LAT-1 expression in transformed hepatic cell lines is not similarly responsive to media amino acid concentrations. In transformed cells LAT-1 is constitutively expressed[15–16]. These observations indicate that LAT-1 expression may confer a growth and survival advantage under the limited amino acid availability that accompanies rapid tumor growth.

During early embryonic development amino acids are fundamental regulators of cell function. In culture medium, amino acids stimulate the attachment and invasion of mouse blastocysts [17]. It has also been shown that active transport of amino acids is required for successful implantation and placentation. The external presence of amino acids however is not sufficient [18]. The placenta is a highly proliferative, highly invasive tissue and its growth is highly regulated. As such, studies of the mechanism(s) regulating placental invasion have been suggested as a model for tumor invasion [19]. We hypothesize that LAT-1 plays an important role in embryo implantation and placentation. In this study our objectives were to analyze qualitatively and quantitatively LAT-1 expression in pre- and post-implantation stages of mouse embryos and to identify the cell types expressing LAT-1 during placental development.

## METHODS

### Embryo collection

Outbred CD-1 female mice (Charles River Laboratory) 5–7 weeks old were induced to superovulate by standard hormonal treatments using an I.P. injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG) followed 48 hr later by an I.P. injection of 7.5 IU of human chorionic gonadotropin (hCG). After overnight mating with males of the same strain, females were inspected for vaginal plugs the next day. This was defined as day 0.5. Thereafter, either oviducts or uterine horns were removed at the stages of development indicated below and in the Results. Embryos were recovered by flushing the oviducts and uterine horns with modified HTF medium with 0.4% bovine serum albumin (BSA). Preimplantation embryos (zygote, 2-cell, 4-cell, morula, blastocyst and hatching blastocyst) were collected at the proper time of

development. Likewise, embryos at E7.5, E9.5 and E11.5 of gestation were obtained by time-dated, natural mating of CD-1 animals. On the appropriate day following the detection of a vaginal plug, uteri with implantation sites were removed. The uterus was cut between each implantation site and embryos were either snap frozen in liquid nitrogen for LCM or fixed and dehydrated with graded sucrose solutions for imunohistochemistry and in situ hybridization. All tissues were stored at −80°C.

## Cryostat Sectioning

Embryos were embedded in OCT medium (Tissue Tek) using peel-a-way disposable embedding molds (Thermo Electron Corporation). Ten-micron sections were prepared in the cryotome (Thermo Electron Corporation). For immunohistochemistry and *in-situ* hybridization 10 μm sections were mounted on *Colorfrost*™ plus slides and for LCM 10 μm sections were mounted on *Colorfrost*™ uncharged slides and stored at −80°C.

## Laser Capture Microdissection (LCM)

Isolation of cells by LCM was performed with minor modifications of the manufacturer's instructions (*Arcturus*™, Mountain View, CA). The frozen embryo sections were thawed at room temperature, fixed in 70% ethanol and stained with an abbreviated hematoxylin and eosin staining protocol for LCM. Slides were cleared with xylene for 5 min and then air-dried and stored in a desiccator. LCM was performed the same day under a microscope attached to a *Pixcell II*™ laser capture microscope using the following settings: excitation wavelength, 495 nm ; laser power, 35–45 milliwatts; duration, 2.5 ms, and laser spot size, 15 μm. Each *CapSure*™ HS LCM cap was used to capture about 100 cells (100 laser shots) from indicated regions of the embryo or placenta. Caps were placed into tubes containing 100 μL of denaturing buffer and stored at −80°C.

## RNA Extraction and Reverse Transcription

For pre-implantation embryos and LCM samples, RNA was isolated using the Micro RNA Isolation Kit (*Stratagene*, La Jolla, CA) that uses guanidinium isothiocyanate to lyse cells and inactivate nucleases. This method was carried out according to the manufacturer's instructions. In pre-implantation samples used for quantitative Real-Time PCR analysis, 0.5 pg/embryo of exogenous Luciferase Control RNA (Promega Corporation, Madison, WI, USA) was added to each pool of embryos prior to RNA extraction.

First-strand complementary DNA (cDNA) synthesis was performed using random hexamers and the *SuperScript*™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

## PCR

Pre-implantation stages of mouse development (oocyte, zygote, 2-cell, 4-cell, 8-cell, morula, blastocyst and hatching blastocyst) and post-implantation LCM samples of various regions of the developing embryo (EPC, GC's, labyrinthine zone, spongiotrophoblast, decidua, and epiblast) were amplified with real-time PCR TaqMan gene expression assays on demand (LAT-1 Assay Cat.# Mm00441516_m1 and GAPDH Cat. # 4352339E-0506006). For pre-implantation embryos, data was normalized to exogenous Luciferase control (ABI TaqMan Left Primer # 185628791-1, Right Primer # 185628791-2, Vic Probe # 185632673-1) and, for post-implantation LCM samples, data was normalized to endogenous GAPDH. The Delta Delta CT method was used for quantitation of mRNA.

Conventional PCR for amplification of LAT-1 was carried out with the following primers:Forward 5′-GGGCACTACCATCTCAAAGTCAGG-3′; Reverse 5′-TTCGTCAGCACATAGACCAGGGTG-3′ (573 bp product with annealing temp=60°C).

## Tissue Preparation and in situ hybridization

Implantation sites (E7.5, E9.5, and E11.5) were dissected in phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C. Tissues were rinsed three times with PBS (5 min) before going through graded sucrose solutions (10% and 25% in PBS) overnight. Tissues were then embedded in the OCT (Tissue Tek) and stored at −80°C. Ten micron sections were cut on a cryostat (Thermo Electron Corporation), mounted on Super Frost Plus slides and stored at −80°C. *In situ* hybridization was carried out with two methods. For Pl-1 (Placental Lactogen 1), Plf (Proliferin) and Tpbpα (Trophoblast specific protein alpha) probes, sections were re-hydrated in PBS, post-fixed in 4% PFA for 10 min, treated with proteinase K (15 μg/mL) for 10 min at room temperature, acetylated for 10 min (acetic anhydride, 0.25%) and hybridized with digoxigenin-labeled probes overnight at 65°C. Digoxigenin labeling was done according to the manufacturers instructions (Roche). Hybridization buffer contained 1× salts (200 mM sodium chloride, 13 mM tris, 5 mM sodium phosphate monobasic, 5 mM sodium phosphate dibasic, 5 mM EDTA), 50% formamide, 10% dextran sulfate, 1 mg/ml yeast tRNA (Roche), 1× Denhardt's (1% w/v bovine serum albumin, 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone), and DIG-labeled probe (final dilution of 1:2000 from reaction with 1 μg template DNA). Two 65 °C post-hybridization washes were carried out (1× SSC, 50% formamide, 0.1% tween-20) followed by two RT washes in 1×MABT (150 mM sodium chloride, 100 mM maleic acid, 0.1% tween-20, pH 7.5), and 30 min RNAse treatment (400 mM sodium chloride, 10 mM tris pH7.5, 5 mM EDTA, 20 μg/ml RNAse A). Sections were blocked in 1×MABT, 2% blocking reagent (Roche), 20% heat inactivated goat serum for 1 h, and incubated overnight in block with anti-DIG antibody (Roche) at a 1:2500 dilution. After four 20 min washes in 1× MABT, slides were rinsed in 1× NTMT (100 mM NaCl, 50 mM MgCl, 100 mM tris pH 9.5, 0.1% tween-20) and incubated in NBT/BCIP in NTMT according to the manufacturer's instructions (Roche). Slides were counterstained with nuclear fast red, dehydrated and cleared in xylene, and mounted in cytoseal mounting medium. For LAT-1 and PolydT probes, sections were re-hydrated in PBS, post-fixed in 4% PFA for 10 min and hybridized to GeneDetect GreenStar*Digoxigenin (DIG)-hyperlabeled oligonucleotide probe according to the manufacturer's protocol (GeneDetect.com/Laboratory Methods). Slides were counterstained with nuclear fast red, dehydrated, cleared in xylene, and mounted in cytoseal mounting medium. All sections were viewed and photographed using an up-right microscope (Nikon Eclipse 80i).

## Immunocytochemistry

Ten micron sections of E7.5, E9.5, and E11.5 mouse embryos were washed 3X with PBS (5 min each wash) and blocked for one hour in room temperatures with 5% goat serum, 1% BSA in PBS. Sections were incubated with 1° LAT-1 polyclonal rabbit antibody (Capralogics, Inc Cat # P00801) overnight at 4°C in the humidified chamber. They were then washed 3X with PBS (5 min each wash) and once in 1% BSA in PBS (5 min), incubated with biotinylated secondary antibody for 1 hour, washed 3X in PBS (5 min each wash), incubated in ABC solution (Vectastain Elite ABC Kit, Vector Laboratories, CA) for 30 min and washed 3 X in PBS (5 min each wash). The sections were then developed color in 0.05% diaminobenzidine (DAB) in PBS for 2 min. The peroxidase reaction was stopped by rinsing the slides in running tap water. The slides were dehydrated, cleared in xylene, and mounted in *Cytoseal*™ mounting medium. Sections were viewed and photographed on a Nikon Eclipse 80i microscope.

### Statistical Analysis

All results are presented as the mean ± S.E. Data represent results from at least three independent experiments. Statistical significance was evaluated using a one-way ANOVA and/or t-test where appropriate. Differences were considered significant when $p \leq 0.05$.

## RESULTS

### LAT-1 expression in pre-implantation stages of mouse embryo development

Conventional PCR was used to confirm LAT-1 expression in preimplantation samples. The stages tested included zygote, 2 cell, 4 cell, morula, blastocyst, and hatching blastocyst (Fig. 1A). LAT-1 mRNA was detected in all stages of pre-implantation mouse embryos tested. Quantitative analysis of LAT-1 mRNA expression in pre-implantation stages of mouse embryo was carried out using real time PCR. Data were normalized to exogenous Luciferase control and reported using the Delta Delta Ct method (Fig. 1B). The results show LAT-1 mRNA at all stages of embryo as it developed from zygote to day 5 blastocyst with highest expression at the hatching blastocyst stage.

### LAT-1 expression in post-implantation stages of mouse embryo development Laser capture microdissection and Real Time PCR analysis

Three post- implantation developmental stages were selected for evaluation of cell specific LAT-1 expression using laser capture, E7.5, E9.5 and E11.5. At embryonic day 7.5 four cell types were microdissected: ectoplacental cone (EPC), epiblast (E), trophoblast giant cells (TGC's) and maternal decidua (D). All gene expression results were normalized to the house keeping gene GAPDH. Quantitative analysis of mRNA expression in these captured regions showed the highest expression of LAT-1 in trophoblast giant cells, followed by maternal decidua, ectoplacental cone and epiblast (Fig. 2A). At embryonic day 9.5 and 11.5 regions were microdissected as follows: labyrinthine zone (L), spongiotrophoblast (S), trophoblast giant cells (TGC's), epiblast (E) and maternal decidua (D). Quantitative analysis of LAT-1 mRNA levels showed expression in each of these regions. By those stages of development there was no difference among cells or regions analyzed (Fig. 2B).

### In-situ hybridization

*In situ* hybridization was used to evaluate regional and cell specific expression of LAT-1 at different stages of embryonic development. Consistent with the laser capture data, differential LAT-1 mRNA expression was only seen at E 7.5 with darkest staining in the trophoblast giant cells. At this stage of development the intensity of hybridization was followed by maternal decidua, ectoplacental cone and epiblast (Fig. 3C). The markers for giant cells (Pl1 and Plf) and marker for spongiotrophoblast cells (Tpbpa) are not seen at this stage of development which is consistent with other observations. By E9.5 and E11.5 LAT-1 expression was no longer preferentially localized to TGCs and hybridization was equal across the different cell types and regions (Fig. 4 and Fig. 5). At those stages both Pl1 and Tpbpa mark the appropriate cell types [20].

### Immunohistochemistry and immunofluorescence

Tissue protein expression level of LAT-1 was assessed using immunohistochemical and immunofluorescent staining. The results are shown in Figure 6. Both immunohistochemistry and immunofluorescence showed the same results (therefore only immunohistochemistry is shown). As was seen with the results from LCM and *in situ* hybridization, LAT-1 protein expression showed the highest intensity of staining in trophoblast giant cells. This was particularly apparent at E7.5 but was also evident at E9.5 and E11.5.

## DISCUSSION

The placenta is the first complex mammalian organ to form during embryogenesis and its function is crucial to successful pregnancy. It is composed of many specialized, trophoblast cell types, each having a particular function and pattern of gene expression [21]. All trophoblast cell types are derived from the trophectoderm, which forms an outer shell of cells surrounding the inner cell mass (ICM) at the blastocyst stage of development. The cells of mural trophectoderm, not in direct contact with ICM, stop dividing and differentiate into trophoblast giant cells (TGC's) that line the implantation site and invade the maternal decidua. In contrast, the cells in direct contact with ICM, polar trophectoderm, continue to proliferate and give rise to the trophoblast cell types that form the placenta [21].

Over expression of LAT-1 is characteristic of many primary human cancers and may be related to tumor progression [12–14]. Its expression has been marked immunohistochemically, correlated with survival and it may respresent a therapeutic target [22,23] The placenta has been employed by many investigators as a valuable experimental model for cancer biology. Like cancer, trophoblast cells are highly proliferative and can express and invasive phenotype. We sought to correlate the expression of LAT-1 with these properties. We examined ths cell specific and temporal expression of LAT-1 by different trophoblast cell types. Our data show that unlike most other transport systems known to be important in later embryonic evelopment, LAT-1 was expressed in preimplantation stages. In the earliest stage examined after implantation (E7.5) LAT-1 expression was restricted to trophoblast giant cells (TGS's). This result was observed for both mRNA (*in situ* hybridization) and protein expression (immunohistochemistry). Later, when TGC's have already made contact with the uterine epithelium and begun to invade and placentation is more advanced (E11.5), mRNA expression was more widely distributed and the level of differential expression was somewhat reduced. At this stage however, protein expression was still greatest in TGC's. This observation is consistent with enhanced LAT-1 expression in invasive cancers [22,23]

Amino acids are critical not only as nutrients for the mammalian embryo but also as regulators of cell motility during implantation and continued development [25–30]. Trophoblast invasion is controlled by very sophisticated systems that specifically regulate motility, independently of many aspects of trophectoderm (TE) differentiation. Regulated motility confers the ability of developing trophoblast cells to initiate invasion. Amino acid signaling in the embryo is regulated in part through the ambient amino acid concentration in uterine environment [30]. LAT-1 is one of the earliest transporters expressed (Fig 1). Unlike most other transport systems known to be important in later embryonic development [31], only LAT-1 is expressed in preimplantation stages. Amino acid uptake and signaling thus provides one way for uterine and embryo developmental changes to be coordinated to the local environment. Culture of embryos in Eagle's non-essential amino acids and glutamine decreases the time required for the first three cell divisions of mouse embryos [28] and stimulates blastocyst formation *in vitro* [29]. The culture of embryos in Eagle's essential amino acids inhibits a development prior to the 8 cell stage. The same media, however, promotes blastocyst development and cell number when introduced after 8 cell stage [29]. A combination of non-essential amino acids and glutamine before the 8- to 16- cell stages and all amino acids after the 8- to 16-cell stages were found to be the best combination to improve embryo viability in vitro [32]. A similar combination of amino acids has been shown to improve human embryo viability *in vitro* and to increase embryo viability post transfer [33–35]. These findings, thus, have let to a significant decrease in the number of embryos that need to be transferred to achieve pregnancy. Amino acids thus play a critical role in preimplantation embryo development. Less is known, however, about the effect of amino acids and their transporters post embryo implantation and during later differentiation.

*In vitro* studies have suggested that embryo implantation is also regulated by the availability of amino acids. Embryos cultured in medium lacking amino acids can not form trophoblast cell outgrowths on fibronectin (an *in vitro* model of implantation) [18]. However, these embryos remain viable for up to 3 days in culture and can be reactivated to form outgrowths upon transfer to complete medium. Also, the window for trophoblast activation by amino acids is very precise during the development. Prior to 120 h post-hCG, alteration of amino acid concentration in culture does not affect later trophoblast outgrowth, whereas after 120 h post-hCG blastocyst will not progress to implantation –competent state unless provided with exogenous amino acids. However, contact with amino acids is needed for only a 4- to 8-h period at 120 h post-hCG. Further exposure to amino acids is not needed for development of trophoblast motility [18]. Amino acid signaling thus acts as a developmental checkpoint.

Amino acid dependent regulation of intracellular signaling pathways has been described in many systems [36]. Amino acid signaling activates the serine-threonine kinase mammalian target of rapamycin (mTOR), which then phosphorylates at least two proteins involved in regulation of translation initiation, p70S6K and PHAS-I [35]. The initiation of trophoblast cell motility also depends on amino acid signaling through mTOR. Treatment with rapamycin, a specific inhibitor of mTOR, inhibits initiation of trophoblast motility and spreading behavior. Rapamycin also blocks amino acid –initiated trophoblast motility and spreading behavior, whereas competitive inhibition of rapamycin with FK506 restores the amino acid stimulation. Under conditions of amino acid deprivation or rapamycin treatment, p70S6K remains un-phosphorylated, confirming that mTOR activation is inhibited in both cases [18]. These results show that amino acid-dependent mTOR signaling is involved in the development of trophoblast cell motility and initiation of implantation.

Since intracellular amino acids are not sufficient to activate mTOR signaling, *de novo* transport of amino acids, and particularly of leucine, is necessary for this activation. It has been proposed by Martin and Sutherland that the activity of the broad-scope and yet leucine- selective amino acid transport system $B^{0,+}$ could produce such increases in intracellular amino acid concentrations. System $B^{0,+}$ uses a $Na^+$ gradient to drive amino acid uptake, and the $Na^+$ concentration in uterine secretions increases by nearly two-fold about 18hrs before implantation. The resultant mTOR signaling could trigger polyamine, insulin-like growth factor II, and nitric oxide production in blastocysts and the increased cell motility which is sometimes associated with synthesis of these bioactive molecules [18].

Mouse blastocysts express at least 14 amino acid transporters. In the preimplantation embryos the activities of at least half of these transporter systems increase significantly upon blastocyst formation [29]. Of note, the $B^{0,+}$ transport system is *not* expressed in preimplantation stages. Our data show that LAT-1 mRNA expression uniquely increases in early stages as the embryo develops to form blastocyst. Given its earlier expression than other transport systems, we believe it is likely that the LAT-1 transporter plays an important role in preimplantation embryo development and subsequent implantation. As mentioned above further studies examining its role in trophoblast motility will provide us with more insights on the mechanism of embryo implantation.

In summary, our results demonstrate that LAT-1 is expressed in all stages of the pre-implantation embryo. We further demonstrate quantitative changes expression as the embryo develops from zygote to blastocyst. Our data show that the cells expressing the highest level of LAT-1 at both the protein and RNA level during early post-implantation stages are TGCs. Trophoblast giant cells, which arise from the mural trophectoderm cells of the blastocyst, transform at the time of implantation into invasive cells that displace and phagocytose the uterine epithelial cells, penetrate the uterine stroma, and make vascular connections with the maternal blood supply. Our findings suggest that LAT-1 may play a critical role in trophoblast

GCs migratory phenotype. Further studies investigating the migration of trophoblast cells using gain of function and/or disruption of LAT-1 expression will reveal its role in the trophoblast cell motility and invasion.

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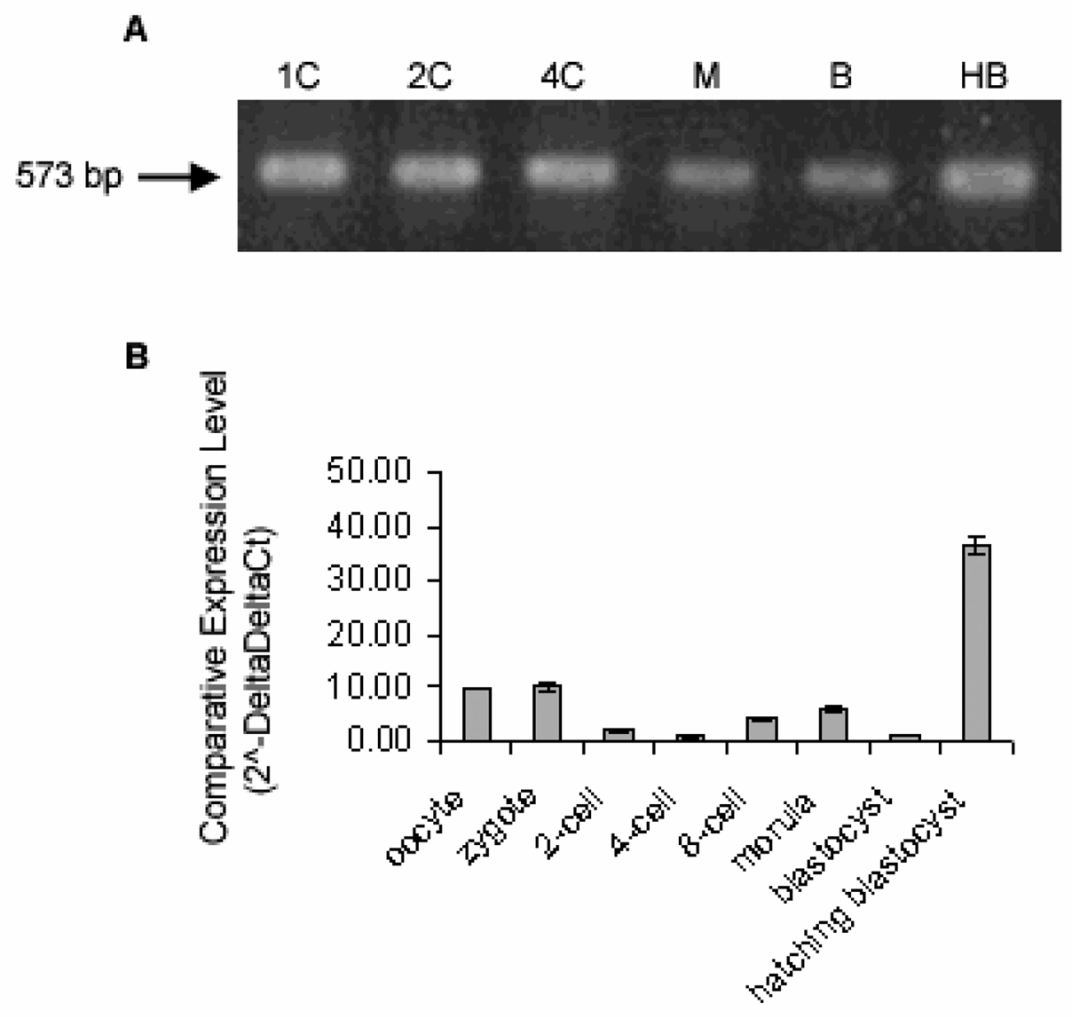


**Fig 1. Detection of mRNA Transcripts encoding LAT1 during Mouse Preimplantation Development**

RT-PCR product encoding LAT1 was detected in cDNA from one (I) embryo equivalent at timed stages of development 1C, 1-cell; 2C, 2-cell; 4C, 4-cell; 8C, 8-cell; M, morula; B, blastocyst; HB, hatching blastocyst **(A).** Representative image of three independent replicates. Quantitative analysis of LAT1 are shown in **(B).** mRNA transcript levels in a developmental series of mouse preimplantation embryos by Real-Time RT-PCR. Data were normalized to external Luciferase control (0.5pg/embryo) and relative to 4-cell target gene mRNA levels. Relative mRNA levels are presented as the mean ± s.e, representative of three independent replicates. Bars with different letters represent significant differences in relative mRNA levels between embryo stages ($p \leq 0.05$).

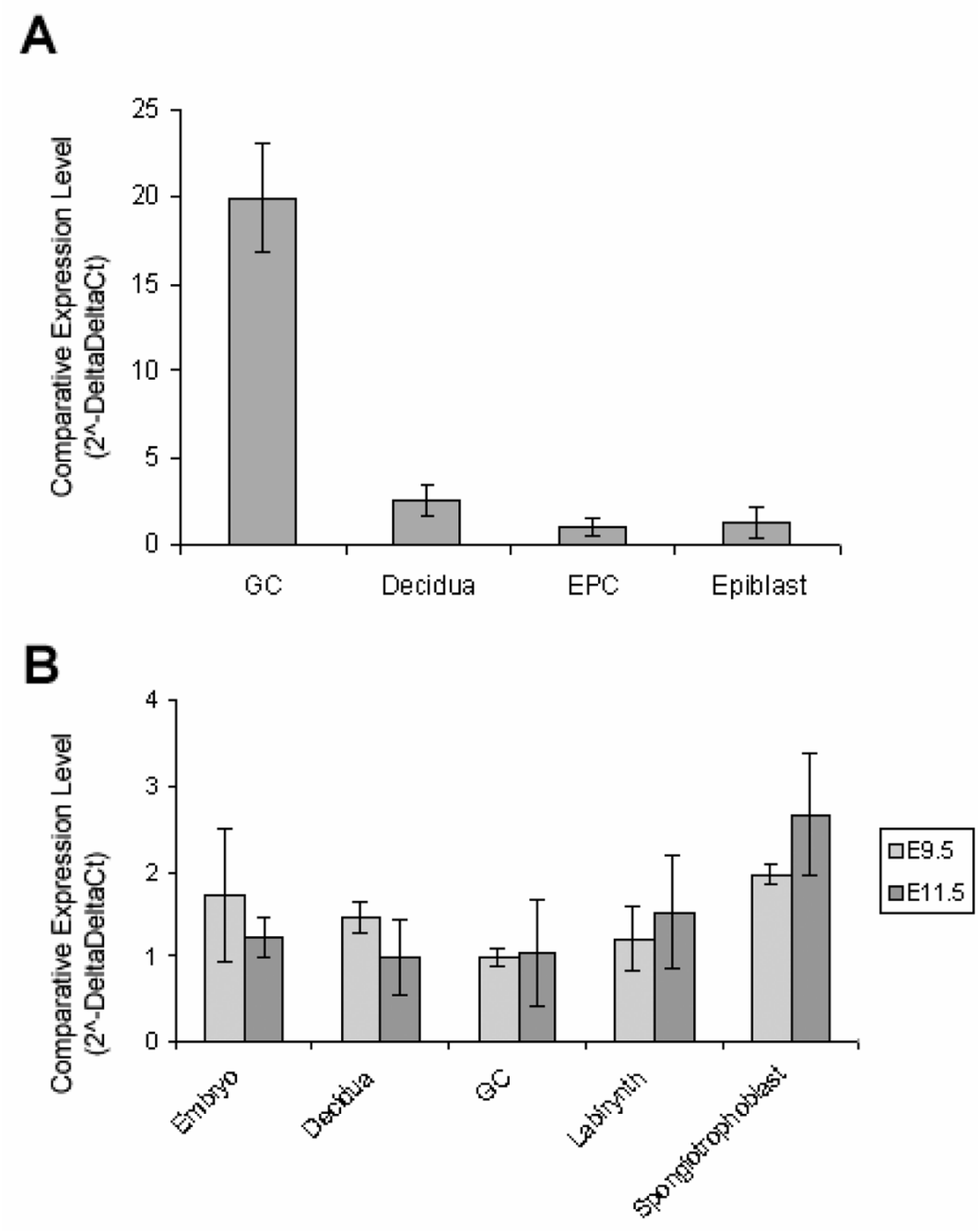


**Fig. 2. Detection of mRNA Transcripts Encoding LAT1 at Early Stages of Mouse Embryo and Placental Development**

mRNA transcript levels at E7.5 **(A)**, E9.5 and E11.5 **(B)** in laser captured samples of mouse embryo and placenta by Real-Time RT-PCR. Data was normalized to GAPDH and relative to EPC for E7.5 and GC's for E9.5 and E11.5 target gene mRNA levels. Relative mRNA levels are presented as the mean ± s.e. representative of three independent replicates. Bars with different letters represent significant differences in relative mRNA levels between tissues ($p \leq 0.05$).

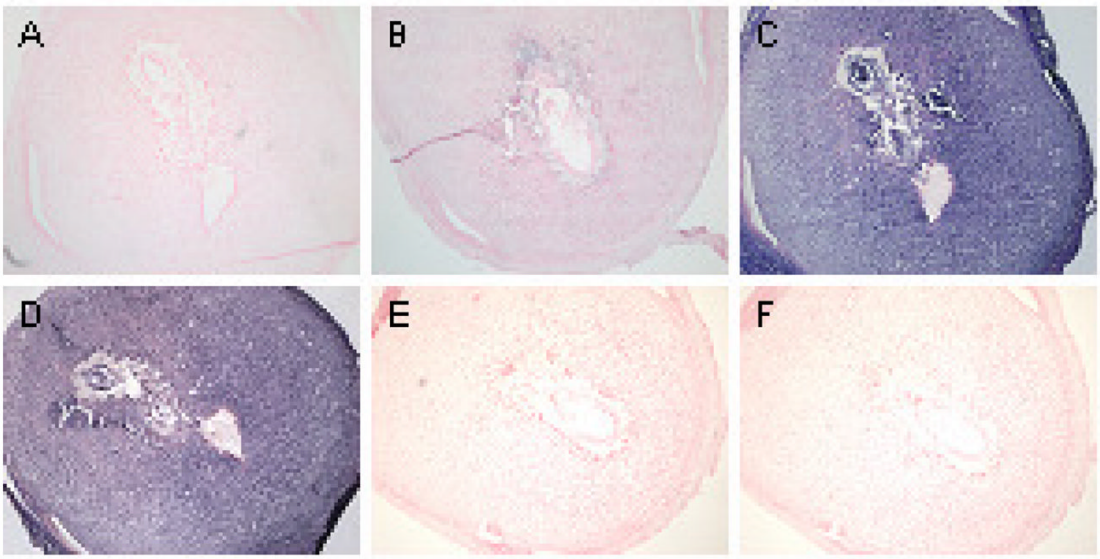


**Fig. 3. In situ hybridization of LAT-1 in E7.5 stage of mouse embryo and placenta**

No probe **(A)**, sense probe for LAT1 **(B)**, anti-sense probe for LAT1 **(C)**, anti-sense probe for PolydT **(D)**, anti-sense probe for Pl1 **(E)**, and anti-sense probe for Tpbpα **(F)**. TGC's lining the implantation site, separating the maternal decidua from the ectoplacental cone as well as ectoplacental cone itself express LAT1 at highest level **(C)** compared to other tissues. All negative controls **(A, B, E, F)** and positive control **(D)** show no staining and homogenous staining across all tissues, respectively. Markers for TGC's such as Pl1 **(E)** and Plf (not shown) are not expressed at that stage of embryo development.

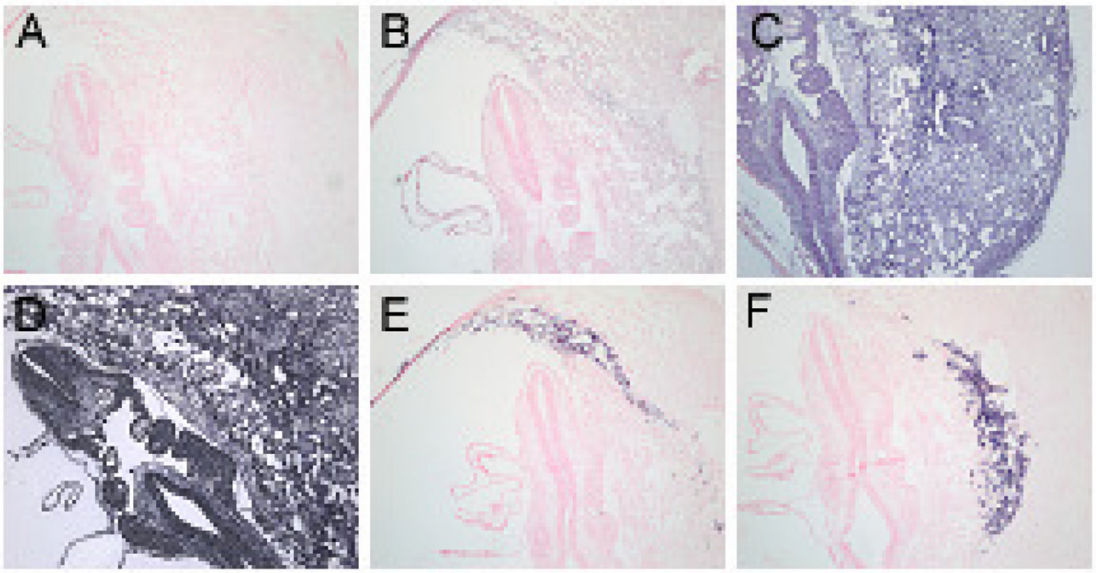


**Fig. 4. In situ hybridization on E9.5 stage of mouse embryo development**

No probe **(A)**, sense probe for LAT1 **(B)**, anti-sense probe for LAT1 **(C)**, anti-sense probe for PolydT **(D)**, anti-sense probe for Pl1 **(E)**, and anti-sense probe for Tpbpα **(F)**. More homogenous expression of LAT1 is seen at E9.5 embryo development with slightly darker staining for TGC's lining the implantation site **(C)**. All negative controls **(A, B)** show no staining and positive controls **(D, E, F)** show either homogenous staining across all tissues for PolydT (D), or TGC specific staining for Pl1 **(E)** and Plf (not shown) and Spongiotrophoblast specific staining for Tpbpα **(F)**.

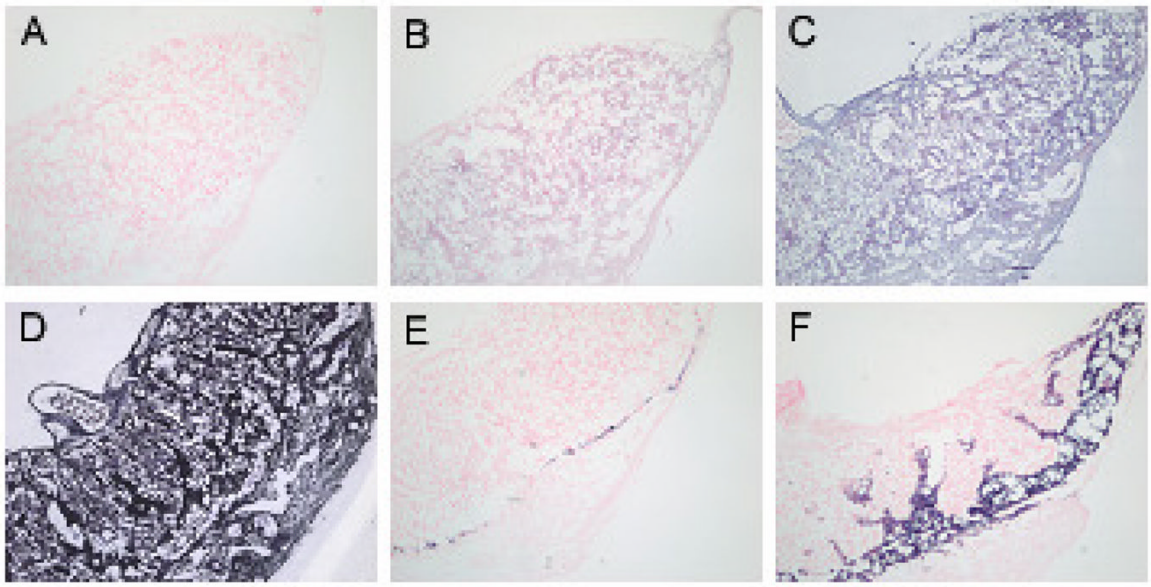


**Fig 5. In situ hybridization on E11.5 stage of mouse embryo development**

No probe **(A)**, sense probe for LAT1 **(B)**, anti-sense probe for LAT1 **(C)**, anti-sense probe for PolydT **(D)**, anti-sense probe for Pl1 **(E)** and anti-sense probe for Tpbpα **(F)**. Homogenous expression of LAT1 is seen at E11.5 embryo development **(C)**. All negative controls **(A, B)** show no staining and positive controls **(D, E, F)** show either homogenous staining across all tissues for PolydT **(D)**, or TGC specific staining for Pl1 **(E)** and Plf (not shown) and spongiotrophoblast specific staining for Tpbpα **(F)**.

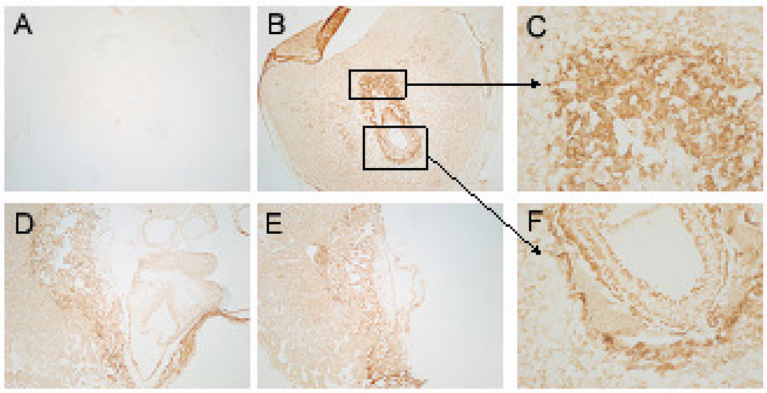


**Fig 6. Immunohistochemichemical Detection of LAT1 at Early Stages of Mouse Embryo Development**

No 1°Ab control **(A)**, LAT1 at E7.5 **(B, C, F)**, LAT1 at E9.5 **(D)** and LAT1 at E11.5 **(E)**. Very distinct expression pattern was seen at E7.5 with highest protein localization in TGC's and ectoplacental cone **(B)**. (4X) Higher power of magnification of TGC's and ectoplacental cone is shown in **(C)** and **(F)**, respectively. (20X) At E9.5 **(D)** and E11.5 **(E)** LAT1 protein is more uniformly distributed among all tissues although TGC's still show highest protein localization.