PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2010, 37(12): 1339~1345
www.pibb.ac.cn

初次卵裂时间是猪克隆胚胎发育潜能的重要标识 *

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摘要 初次卵裂时间与哺乳动物胚胎发育潜能有关．比较了不同初次卵裂时间(20～24 h，早期；25～36 h，中期；37～48 h，晚期；20～48 h，对照)的猪孤雌(parthenogenetic，PA)、体细胞核移植(somatic cell nuclear transfer，SCNT)胚胎的囊胚发育率、扩张囊胚发育率和囊胚细胞数，评价其体外发育能力．发现早期卵裂的 PA 胚胎发育到第 6 天的囊胚发育率显著高于中期、晚期以及对照组($P < 0.05$；54.0% *vs*. 19.6%，5.4%，18.7%)．扩张囊胚发育率，早裂胚胎同样优于其他组．早期卵裂的 SCNT 胚胎发育到第 6 天的囊胚比率高于中期卵裂胚胎(32.2% *vs*. 23.5%)，而晚期卵裂胚胎发育到囊胚的比率最低(6.3%)．早期卵裂的 SCNT 胚胎发育到第 6 天的扩张囊胚比率显著高于其余各组 ($P < 0.05$；18.9% *vs*. 5.9%、3.1%、7.4%)．囊胚细胞数在早期、中期、晚期三组之间表现出下降趋势．将早期卵裂的 SCNT 胚胎与未经挑选的对照组胚胎分别进行移植，观察其体内发育能力．移植早裂 SCNT 胚胎的受体在产仔数和克隆效率上均明显高于未经挑选胚胎的受体(4.7 *vs*. 2.1；3.9% *vs*. 0.9%)，说明早裂胚胎着床后具有更强的发育能力．以上结果表明：初次卵裂时间可以作为猪克隆胚胎发育潜能的重要标识，选择早裂的胚胎进行移植，有助于提高克隆效率．

关键词 猪，体细胞核移植，初次卵裂时间，发育潜能
学科分类号 Q813.7
DOI: 10.3724/SP.J.1206.2010.00115

世界首例体细胞克隆猪在 2000 年诞生[1–3]．随后，人们又利用体细胞克隆技术生产转基因猪[4–5]、基因组修饰(如基因打靶)猪等[6-8]．但是，克隆效率一般在 1%左右，仅少数几个实验组的克隆效率超过了 1%[9–11]．为了提高克隆效率，克隆技术的很多环节都进行了不断改进，包括供体细胞的选择及其同期化[12–13]、卵母细胞的体外成熟条件[14]、供体细胞与卵母细胞的融合、激活条件[15–16]、胚胎培养环境[17]、胚胎移植时期及受体的选择[18–19]等．

目前，导致克隆猪效率较低的主要原因之一是移植的克隆胚胎当中优质胚胎(形态学上观察，第 6 天囊胚扩张比较明显，如图 1 和图 2 所示)数量太少．而猪作为多胎动物，至少需要 4 枚以上有效的胚胎进行移植[20–21]．当前判定着床前胚胎的质量，主要从形态学、卵裂率、囊胚和扩张囊胚发育率及囊胚细胞数等指标进行衡量．然而，猪体细胞克隆胚胎移植一般在 1～2 细胞阶段[1, 4, 11, 19]，针对这一早期阶段，目前没有一个有效的评价方法来预测早期胚胎的发育潜能．考虑到当前体外培养条件下，克隆胚胎发育能力较差的原因，每头受体母猪需移植 100 枚以上的克隆胚胎，确保其妊娠维持[8, 10–12]．我们在猪克隆胚胎体外培养的过程中发现：猪克隆胚胎体外培养初次卵裂(2 细胞期)的时间是不一致的，从激活后 20 h 左右，部分胚胎开始分裂成 2 细胞，直到 48 h 前后完成首次卵裂．整个胚胎体外培养到第 6 天，发现不同的囊胚在直径和孵化状态上有很大的差异．

* 北京市农业科技项目(20090612)和国家高技术研究发展计划(863)(2008AA101003)资助项目．
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收稿日期：2010-05-11，接受日期：2010-08-25

为了评价早期阶段克隆胚胎的后期发育能力，以便挑选出优质的胚胎，从而减少克隆胚胎移植的数量，整体上提高克隆效率，本研究探索了猪克隆胚胎初次卵裂时间与其发育潜能的关系.

1 材料与方法

除特别注明外，所有化学试剂均购自 Sigma-Aldrich 公司；细胞培养相关耗材为 BD Falcon 公司产品；卵母细胞及胚胎培养耗材为 Nunc 公司产品.

1.1 实验设计

将猪孤雌(parthenogenetic，PA)和体细胞核移植(somatic cell nuclear transfer，SCNT)胚胎分别进行体外培养，根据不同胚胎初次卵裂的时间先后顺序，在激活后 24 h 挑选出发生卵裂的胚胎，并将其转移到新的培养滴中继续培养，剩余未分裂胚胎在激活后 36 h 和 48 h 重复上述过程，以 0～48 h 未分时间段挑选的胚胎作为对照组，待胚胎发育到第 6 天时，比较了激活后初次卵裂时间不同(早裂，20～24 h；中裂，25～36 h；晚裂，37～48 h；对照，20～48 h)胚胎的体外发育能力(囊胚率、扩张囊胚率和囊胚细胞数). 为了进一步探索 SCNT 胚胎在体内的发育能力，将早裂与对照的 SCNT 胚胎分别移植到自然发情的母猪体内，最终确定初次卵裂时间与猪克隆胚胎发育潜能的关系.

1.2 卵母细胞体外成熟(*in vitro* maturation，IVM)

从屠宰场采集初情期前母猪卵巢，放入含青霉素、链霉素的 35℃ 生理盐水中，2 h 内运回实验室. 用配有 18 号针头的 10 ml 注射器抽吸卵巢上 3～6 mm 的卵泡. 体视镜下挑选卵丘包裹 3 层以上、致密、胞质均匀的卵丘细胞 - 卵母细胞复合体(cumulus- oocyte-complexes，COCs)，卵母细胞体外成熟液(IVM 液)为 NCSU-23 添加 10%猪卵泡液(PFF)、0.57 mmol/L 半胱氨酸、10 μg/L 表皮生长因子 (EGF)、10 U/ml 人绒毛膜促性腺激素(hCG)和 10 U/ml 孕马血清促性腺激素(eCG)，培养条件为 38.5℃ 、5% CO_2、饱和湿度环境. 将 COCs 先在 IVM 液中培养(20±2) h，之后转移到无 hCG，eCG 的 IVM 液中继续培养(20±2) h. 在 IVM 中 40～44 h 后，将 COCs 转移到含 1 g/L 透明质酸酶中脱去卵丘细胞，挑选排出第一极体、卵黄膜完整、卵周隙清晰、胞质均匀的 MⅡ卵母细胞为受体.

1.3 供体细胞的建立

从北京浩邦猪人工授精有限责任公司挑选出 1 头成年优秀的种公猪，无菌操作取耳部组织，采用常规组织块接种培养建立细胞系，原代细胞采用 DMEM(Gibco 公司) 添加 20%胎牛血清贴壁培养，传代细胞采用 DMEM 添加 10%胎牛血清培养，培养条件为 37.0℃ 、5% CO_2、饱和湿度的环境，细胞在体外经 3～5 次传代用于核移植.

1.4 卵母细胞孤雌激活

将 MⅡ卵母细胞转移到已经铺满激活液，电极宽度为 0.5 mm 的融合槽(BTX, USA)中，用 BTX 细胞融合仪施加 2 个 2.0 kV/cm、30 μs 的直流脉冲(DC)诱导激活. 洗涤后将卵母细胞转移到胚胎培养液 PZM-3 +10 mg/L 细胞松驰素 B(cytochalasinB，CB)的液滴内作用 4 h，然后转移到胚胎培养液 PZM-3 内培养到第 6 天观察囊胚发育状况.

1.5 体细胞核移植

采用显微操作结合 spindle-view 系统去核和电融合法进行体细胞核移植，过程简述如下:显微操作液为添加 7.5 mg/L 细胞松驰素 B(CB)的 HEPES 缓冲的无钙 NCSU-23. 在 spindle-view 系统下去除 MⅡ 卵的纺锤体及第一极体，选取一个体细胞注射到透明带下，并使之与卵母细胞质膜紧密接触，用 ECM2001 融合仪(BTX)施加 1 个 2.0 kV/cm、30 μs 的直流电脉冲诱导融合并同时激活，融合 / 激活液为 0.28 mol/L 甘露醇、 0.1 mmol/L $CaCl_2$、0.1 mmol/L $MgCl_2$、0.5 mmol/L 的 HEPES 及 0.01% PVA 组成. 融合的重构胚放入含 10 mg/L CB 和 10 mg/L 放线菌酮(cyclohexmide，CHX)的胚胎培养液 PZM-3 溶液中进行 2 次激活处理 4 h，最后转入 PZM-3 内进行培养，体外观察胚胎发育到第 6 天的囊胚状况或进行早期移植.

1.6 囊胚细胞计数

将第 6 天的囊胚取出，用 DPBS-PVA 洗涤 3 遍，4%多聚甲醛固定 10 min，再用 DPBS-PVA 洗涤 3 遍, 转移到 5 mg/L Hoechst 33342 的 DPBS-PVA 中染色 10 min, 压片，荧光显微镜(BX51, Olympus)下进行细胞计数.

1.7 胚胎移植和妊娠检测

克隆胚胎体外培养 1～2 天后，挑选形态和发育较好的胚胎进行移植，自然发情第 1 天或第 2 天的后备母猪用作受体(以出现压背静立反射为发情的第 0 天)，移植方法为手术法输卵管深部移植.

试验组母猪每头受体移植约 100 枚 20～24 h 挑出发生分裂的胚胎，对照组母猪每头受体移植 200 多枚混合胚胎(未分时间段进行筛选). 胚胎移

植后 40 天，进行超声波妊娠检测.

1.8　微卫星鉴定

常规方法提取体细胞、克隆猪耳组织和代孕母猪的耳组织 DNA，选取位于不同染色体上的 6 对分布于不同染色体荧光标记的微卫星多态性位点作为遗传标记，进行遗传一致性的检测，按优化的反应条件对上述基因组 DNA 进行 PCR 扩增，PCR 扩增产物在扩增后通过 ABI 9700 自动荧光测序仪凝胶电泳，Peak Scanner 软件进行图像收集和分析，计算出微卫星等位基因片段大小. 整个鉴定过程由上海基康有限责任公司完成.

1.9　统计分析

将初次卵裂时间不同的 PA 胚胎，SCNT 胚胎等体外囊胚发育率、扩张囊胚发育率及囊胚细胞数，使用 SPSS 17.0 进行单因素方差分析.

2　结　　果

2.1　不同初次卵裂时间的 PA 胚胎的体外发育

卵母细胞孤雌激活后卵裂、发育至囊胚及囊胚细胞计数见图 1. 在孤雌胚胎的体外发育中，早期卵裂 PA 胚胎的囊胚发育率明显优于中期卵裂胚胎和晚期卵裂胚胎以及对照组，差异显著($P < 0.05$; 54.0% *vs*. 19.6%，5.4%，18.7%). 扩张囊胚发育率，早裂胚胎同样明显优于其他组，差异显著($P < 0.05$；32.6% *vs*. 5.6%，1.8%，9.7%). 中期卵裂胚胎的囊胚率显著高于晚期卵裂胚胎($P < 0.05$). 早裂、中裂、晚裂以及对照组 4 组胚胎发育到囊胚阶段的囊胚细胞数依次为 54.5 ± 8.7、52.8 ± 7.5、37.2 ± 6.7、48.7 ± 6.9，其中早期卵裂胚胎最高，晚期卵裂胚胎最低，4 组之间无显著差异(表 1).

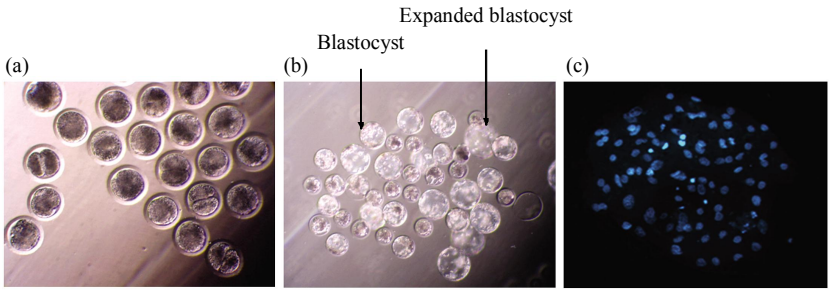

Fig. 1　Development result of parthenogenetically activated embryo

(a)2-cell stage embryos(200×). (b) Blastocysts (6d)(200×). (c) Cells number of blastocyst (400×).

Table 1　*In vitro* development of parthenogenetic embryos related to timing of the first zygotic cleavage

Number of cultured embryos	Timing of first zygotic division	Number of 2-cell embryos	Number (%) of blastocysts[1)]	Number (%) of expanded blastocysts[2)]	Total cell number of blastocysts[3)]
790	20～24 h	298	161 (54.0)[1)]	97 (32.6)[1)]	54.5 ± 8.7
	25～36 h	107	21 (19.6)[2)]	6 (5.6)[2, 3)]	52.8 ± 7.5
	37～48 h	56	3 (5.4)[3)]	1 (1.8)[3)]	37.2 ± 6.7
460	20～48 h	257	48 (18.7)[2)]	25 (9.7)[2)]	48.7 ± 6.9

[1)] Number of blastocysts/Number of 2-cells. [2)] Number of expanded blastocysts/Number of 2-cells. [3)] $\bar{x} \pm s$. Values with different superscripts within a column are significantly different ($P < 0.05$).

2.2　不同初次卵裂时间的 SCNT 胚胎的体外发育

重构胚激活后卵裂、发育至囊胚及囊胚细胞计数见图 2. 在克隆胚胎的体外发育中，早裂胚胎比中期卵裂胚胎的囊胚发育率高(32.2% *vs*. 23.5%)，但差异不显著($P > 0.05$). 然而，晚裂胚胎发育到囊胚阶段的比例较低(6.3%). 针对扩张囊胚发育率，早期卵裂胚胎显著高于中期卵裂胚胎、晚裂胚胎以及对照组($P < 0.05$；18.9% *vs*. 5.9%，3.1%，7.4%). 4 组胚胎发育到囊胚阶段的囊胚细胞数依次为 53.6 ± 8.9、41.9 ± 4.3、35.5 ± 5.5、43.7 ± 4.5，其中早裂胚胎最高，晚裂胚胎最低，但 4 组之间无显著差异(表 2).

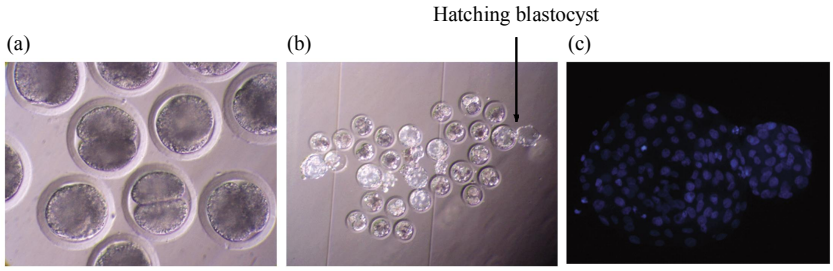

Fig. 2　Development result of somatic cell nuclear transfer embryo

(a) 2-cell stage embryos(300×). (b) Blastocysts (6d)(200×). (c) Cells number of blastocyst (400×).

Table 2　*In vitro* development of cloned embryos with different timing of the first zygotic cleavage

Number of cultured embryos	Timing of first zygotic division	Number of 2-cell embryos	Number (%) of blastocysts[1)]	Number (%) of expanded blastocysts[2)]	Total cell number of blastocysts[3)]
	20～24 h	143	46 (32.2)[1)]	27 (18.9)[1)]	53.6 ± 8.9
359	25～36 h	85	20 (23.5)[1,2)]	5 (5.9)[2)]	41.9 ± 4.3
	37～48 h	32	2 (6.3)[3)]	1 (3.1)[2)]	35.5 ± 5.5
175	20～48 h	122	16 (13.1)[2,3)]	9 (7.4)[2)]	43.7 ± 4.5

[1)] Number of blastocysts/Number of 2-cells. [2)] Number of expanded blastocysts/Number of 2-cells. [3)] $\bar{x} \pm s$. Values with different superscripts within a column are significantly different ($P < 0.05$).

2.3　克隆猪胚胎移植后的体内发育

将 3 437 枚 1～4 细胞期阶段的克隆猪胚胎移植到 18 头自然发情的母猪输卵管内，共产仔 53 头(9 个死胎，3 个木乃伊). 其中 718 枚早期卵裂胚胎分别移植到 6 头受体母猪，产仔 28 头(1 个死胎)，单产最高达 11 头(图 3)，平均每头母猪产 4.7 头，克隆效率为 3.9%. 另外，将 2 719 枚未经挑选胚胎移植到 12 头母猪，产仔 25 头(8 个死胎，3 个木乃伊)，平均每头母猪产 2.1 头，克隆效率为 0.9%(表 3).

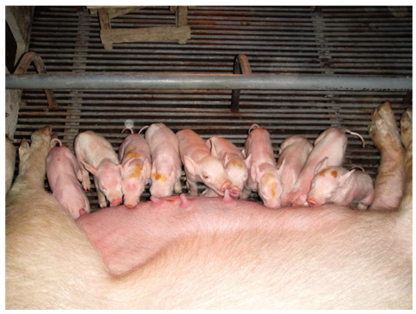

Fig. 3　905001 recipient born 11 somatic cloned piglets in one litter

Table 3　*In vivo* development of somatic cell nuclear transfer embryo

Number of recipient gilts	Embryo status	Transferred embryo(Number)	40d pregnant	Piglet born	Cloning efficiency/%
906005	Early cleavage	110	+	5	4.5
800104	Early cleavage	120	+	7(1 stillborn)	5.8
070828	Early cleavage	98	+	0	0
903018	Early cleavage	108	+	5	4.6
905001	Early cleavage	150	+	11	7.3
809025	Early cleavage	132	+	0	0
081103	No selected	205	+	3	1.4
800124	No selected	209	+	4(2 stillborn, 1 mummified fetuse)	1.9
906006	No selected	200	+	2	1.0
090620	No selected	270	+	0	0
090612	No selected	206	+	0	0
903017	No selected	230	–	0	0
7855	No selected	320	+	0	0
D9002	No selected	162	+	5(2 stillborn)	3.1
6148	No selected	220	+	6(2 mummified fetuses, 1 stillborn)	2.7
090405	No selected	198	+	2(1 stillborn)	1.0
09003	No selected	209	+	3(2 stillborn)	1.4
D9004	No selected	290	–	0	0

Early cleavage: The embryos cleavaged firstly during 20～24 h. No selected: The embryos cleavaged firstly during 20～48 h. Cloning efficiency: Number of piglets/Number of embryos transferred.

2.4 供体细胞、克隆仔猪和受体母猪的微卫星鉴定结果

对供体细胞、53 头克隆猪中存活的 41 个个体及有存活仔猪的 4 头受体母猪进行了遗传鉴定，6 个微卫星位点检测结果见表 4. 结果表明，所有的克隆猪个体均与供核细胞具有相同的微卫星多态性，而与 4 头受体母猪无亲缘关系.

Table 4 Microsatelite analysis performed on six polymorphic loci，comparison of the donor cell line，four surrogate gilt and forty-one cloned piglets

Groups	Sw24	Sw72	Sw857	Sw122	S0070	S0226
Donor cell	96/115	98/98	148/150	110/113	262/262	182/194
Piglets(n=41)	96/115	98/98	148/150	110/113	262/262	182/194
Recipients(1)	96/115	106/108	143/147	110/115	270/277	182/194
Recipients(2)	115/115	98/100	143/143	109/109	293/293	194/200
Recipients(3)	109/115	98/106	139/152	115/117	273/273	182/194
Recipients(4)	110/115	106/108	142/142	109/109	271/271	182/194

3 讨 论

哺乳动物胚胎的初次卵裂时间关系到胚胎的后期发育. 很多研究表明，胚胎培养过程中，早期卵裂胚胎相对于后期晚裂的胚胎更容易发育到囊胚阶段[22-24]. 在人的胚胎移植过程中，2003 年，Salumets 等[25]发现，移植早期卵裂的胚胎(25～27 h)，怀孕率在 50%，而移植晚期卵裂的胚胎，怀孕率在 26.4%. 牛的早期卵裂胚胎发育到囊胚的几率明显高于晚期卵裂胚胎，并有很高的囊胚细胞数、较低的细胞凋亡比例、较低的染色体异常率、玻璃化冷冻后有较高的存活率[26].

在 PA 胚胎发育中，早期卵裂胚胎相对于中期卵裂胚胎和晚期卵裂胚胎以及对照组的囊胚率和扩张囊胚率都有极大的提高，囊胚细胞数也是最高，说明 PA 早期卵裂胚胎整体上有较强的后期发育潜能，优质囊胚的数量最多. 有文献报道：胚胎早期发育过程中，分裂较早的胚胎中线粒体 DNA 的含量比分裂较晚的胚胎中多，而在受精能力较强的精子中，线粒体 DNA 的含量则比较低，证明了胚胎早期发育主要由卵母细胞调控[27]. 有研究认为排除第一极体时间较早的卵母细胞，孤雌激活后发育到囊胚的比例就高，反之就低[28]. 进一步说明初次卵裂时间不仅与胚胎后期发育潜能有关而且与卵母细胞质量有很大关系.

有研究表明，大多数囊胚的形成，是从早期卵裂胚胎发育而来，这些囊胚有很好的扩张和孵化状形态以及囊胚腔维持能力[29]. 本试验中，SCNT 早期卵裂胚胎的囊胚率优于中期卵裂胚胎和对照组，尤其扩张囊胚率有很大提高，囊胚细胞数也相对最高，说明 SCNT 早期卵裂胚胎的后期发育潜能和质量都较好，优质囊胚的数量较多.

克隆猪出生结果表明，移植少量早裂 SCNT 胚胎(98～150 枚)的受体在平均产仔数 (4.7 头 *vs*. 2.1 头) 和克隆效率上 (3.9% *vs*. 0.9%) 均明显高于移植大量未经挑选胚胎(198～320 枚)的受体. 直到现在，有关克隆猪单产最高纪录是在 2008 年，Petersen 等[10]试验中有两头受体分别移植了 121 枚和 80 枚处于 1-cell 阶段的胚胎，单窝各自获得了 11 头和 12 头克隆仔猪. 在我们的移植试验中，有一头受体母猪移植了 150 枚处于 2～4 cell 阶段的早期卵裂胚胎，最终产仔 11 头. 进一步验证了猪克隆早期卵裂胚胎具有良好的后期发育潜能，明显提高了克隆猪效率. 初次卵裂时间可作为猪克隆胚胎发育潜能的重要标识，为猪克隆胚胎发育的分子标识提供试验基础，也为克隆技术及转基因动物育种产业化提供一个重要的技术参考.

初次卵裂是由积累在母源胞质中的转录物来调节[30]. 有关猪克隆胚胎卵裂早晚与后期发育潜能的分子机制，我们已筛选出 2 个在早期卵裂胚胎中丰富表达的基因 *CPEB2* 和 *hnRNPs*，可能与胚胎发育能力相关(未发表)，相关机制正在进一步研究之中.

致谢 感谢北京安定种猪场郭和平总经理和张忠良场长及有关技术人员在受体猪选择和克隆仔猪培育，中国农业大学动物医学院张健博士在猪胚胎移植手术，北京东方联鸣公司王少华工程师在受体母猪妊娠 B 超监测，赵凤有技术员在猪卵巢采集所给予的帮助和支持.

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Timing of The First Zygotic Cleavage as a Developmental Potential Marker for Porcine Cloned Embryos*

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Abstract Timing of the first zygotic cleavage is related to the developmental potential of mammalian embryos. This phenomenon in porcine parthenogenetically activated (PA) and somatic cell nuclear transfer (SCNT) embryos was documented. *In vitro* matured pig oocytes were either activated parthenogenetically or microinjected with a somatic cell, followed by electro-fusion. At 24 h post activation (hpa), PA and SCNT embryos were assessed visually, and cleaved embryos were moved into new culture wells. This process was repeated at 36 hpa or 48 hpa. Embryos in different groups were allowed to develop for 6 days in culture. The relationship between embryo developmental competence and the first zygotic cleavage at different timing (early-cleaving, 20 ～24 h; mid-cleaving, 25 ～36 h; late-cleaving, 37 ～48 h and unselected controls, 20 ～48 h) was evaluated by the proportions of cleaved embryos developed to blastocysts and expanded blastocysts (EB), and blastocyst total cell number. For PA embryos, the proportion of early-cleaving embryos that developed to blastocysts was significantly higher than that of mid-cleaving, late-cleaving and controls ($P < 0.05$; 54.0% *vs*. 19.6%, 5.4% and 18.7%, respectively); a similar pattern was noted for the formation of EB. For SCNT embryos, the proportion of early-cleaving embryos developing to blastocysts was not significantly higher than that of mid-cleaving embryos (32.2% *vs*. 23.5%), but the late-cleaving embryos had poor developmental competence to blastocysts (6.3%). Early-cleaving SCNT embryos showed a significant higher competence developing to expanded blastocysts than the mid-, late-cleaving and unselected control embryos ($P < 0.05$; 18.9% *vs*. 5.9%, 3.1%, 7.4%, respectively). Total cell number in the blastocysts declined across the three cleavage-timed groups. Developmental potential of SCNT cloned embryos was assessed *in vivo* by transferring early-cleaving (< 24 h) and unselected (time of cleavage up to 48 h not determined) embryos into recipient gilts. Litter size and cloning efficiency (piglets born/transferred embryos) in recipients of early-cleaving embryos were significantly higher than those in recipients of unselected embryos (4.7 *vs*. 2.1 piglets; 3.9% *vs*. 0.9%). The data demonstrate that the developmental potential of early-cleaving SCNT embryos is higher than that of later-cleaving embryos, thus suggesting that timing of the first zygotic cleavage can be utilized as a useful parameter for indicating developmental potential of porcine cloned embryos.

Key words pig, somatic cell nuclear transfer, timing of the first zygotic cleavage, developmental potential
DOI: 10.3724/SP.J.1206.2010.00115

*This work was supported by grants from The Agricultural Science and Technology Project of Beijing (20090612) and Hi-Tech Research and Development Program of China (2008AA101003).

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Received: May 11, 2010 Accepted: August 25, 2010